Viral and Host Gene Expression during Human Cytomegalovirus Infection

Evangelia Sofia Gramoustianou

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Department of Virology[1]
Royal Free and University College Medical School
Royal Free Campus
University College London

Wohl Virion Centre[2]
Department of Immunology and Molecular Pathology
Windeyer Institute of Medical Sciences
University College London

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

HCMV infection is usually asymptomatic in immunocompetent hosts, but serious disease can occur in immunocompromised individuals and in congenitally infected newborns. The importance of virus fitness determinations became evident in 1976, when it was proposed that the infecting strain of HCMV is important for clinical outcome, along with the intensity and duration of viral replication. HCMV strains exhibit different levels of virulence *in vivo*, depending on their passage history in cell culture. High and low passage HCMV strains exhibit tropism differences *in vitro*, suggesting that different tissue tropism may occur *in vivo*. In addition, approximately 13kb of novel DNA sequences located near the right edge of the unique long component of the genome has been identified in Toledo and clinical strains. This region (UL/b') encodes several open reading frames, which are missing from the high passage laboratory-adapted variants of Towne and AD169 and are thought to play important roles in pathogenesis.

One of the aims of this thesis was to determine the replication dynamics of different HCMV strains *in vitro* as well as compare their ability to bind to cells and mediate cell-to-cell spread of infection using pair-wise competition experiments in cell culture. AD169 was shown to replicate better than Toledo in fibroblasts. Furthermore, assessment of the replication of Toledo in a different cell type, HUVEC, indicated that the virus replicated to higher levels in fibroblasts. Towne was found to bind to HEL cells with higher affinity compared to AD169. The results showed phenotypic differences between high and low passaged HCMV variants and also illustrated that fitness differences between them are variable and highly dependent upon the status of the virus inoculum. To begin to understand the complex relationship between tissue tropism, virulence and HCMV genome composition, a DNA microarray approach was developed to examine host and HCMV gene expression during the productive infection of two distinct cell lines, fibroblasts and endothelial cells. The results showed that genes within the UL/b' region were expressed in a cell type-specific fashion. In the context of host cell gene expression, cell type-specific host gene transcriptional changes were observed, reflecting different viral modulation of distinct cell type environments. The results provided potential insight into the function of genes encoded in the UL/b' region. Of particular note was that transcriptional changes frequently occurred in genes associated with pathways involved in the pathology of HCMV in the human host.

For my family

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Abbreviations

AFU	antigen forming units
Ag	antigen
AIDS	acquired immunodeficiency syndrome
aRNA	amplified RNA
AMP	adenosine monophosphate
ARF	ADP-ribosylation factor
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
bp	base-pair
CDK	cyclin dependent kinase
cDNA	complementary DNA
CEA	carcinoembryonic antigen
CFU	colony forming units
CMV	cytomegalovirus
COX-2	cyclooxygenase-2
CTL	cytotoxic T lymphocyte
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
CNS	central nervous system
DEAFF	detection of immediate early/early antigen fluorescent foci
DEPC	diethylynene pyrocarbonate
DMSO	dimethyl sulphoxide
DMVEC	dermal microvascular endothelial cell
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate mix
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
ds	double-stranded
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
E.Coli	Escherichia Coli
EST	expressed sequence tag
EtBr	ethidium bromide
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
gB	glycoprotein B
GCV	ganciclovir
gp	glycoprotein
h	hours
HAART	highly active anti-retroviral therapy
HBV	hepatitis B virus
HCMV	human cytomegalovirus
HCV	hepatitis C virus
HEL	human embryonic lung
HHV	human herpesvirus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPLC	high performance liquid chromatography
HPV	human papilloma virus
HSV	herpes-simplex virus
HTLV-1	human T-cell leukaemia virus-1
HUVEC	human umbilical vein endothelial cells
IE	immediate early
IFN	interferon

Ig	immunoglobulin
IL	interleukin
IRF	interferon regulatory factor
IVT	*in vitro* transcription
JAK	janus-activated kinase
kb	kilobase
KSHV	Kaposi's sarcoma-associated herpesvirus
MAPK	mitogen-activated protein kinase
MDV	Marek's disease virus
MEM	minimal essential medium
MHC	major histocompatibility antigen
min	minute
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor of κ light polypeptide gene enhancer in B-cells
NK	natural killer
nt	nucleotide
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming unit
pi	post-infection
pol	polymerase
PSG	pregnancy-specific glycoprotein
QC-PCR	quantitative competitive PCR
RNA	ribonucleic acid
RSV	respiratory syncytial virus
RT	reverse transcriptase
RT-PCR	reverse transcription-polymerase chain reaction
RFLP	restriction fragment length polymorphism
SCID	severe combined immunodeficient
SD	standard deviation
SDS	sodium dodecyl sulphate
sec	second
STAT	signal transducer and activator of transcription
Taq	*Thermus aquaticus*
TNF	tumour necrosis factor
UV	ultra-violet
V	volts

Chapter 1

Introduction

1.0 Human cytomegalovirus characterisation

1.1 Classification

Human cytomegalovirus (HCMV) is classified as a member of the *Herpesvirinae* family, based on virion structure and several biological characteristics, including the ability to establish persistent and latent infections. Herpesviruses are widespread in nature and to date, eight of these have been isolated from humans. They are termed human herpesvirus 1-8 (HHV 1-8), reflecting the order of their identification (Roizman and Pellett, 2001). All herpesviruses are classified into the α-, β- and γ herpesviruses, due to significant variation in biological properties, genome organisation and gene content. The human herpesviruses and the diseases they cause are listed in Table 1.1.

The alphaherpesviruses are rapidly growing viruses with a short replication cycle and a variable host range *in vivo*. HHV-1 (herpes simplex virus 1, HSV-1), HHV-2 (herpes simplex virus 2, HSV-2), and HHV-3 (varicella-zoster virus VZV) belong to this family. HCMV (HHV-5) is classified as a betaherpesvirus due to its slow replication cycle *in vitro*, enlargement of infected cells ("cytomegalic" appearance) and genetic differences (McGeoch *et al*, 1995). Cytomegaloviruses exhibit strict species specificity, although the replication of baboon CMV in human fibroblasts was reported in 1997 (Michaels *et al*, 1997). Other members of the betaherpesviruses include HHV-6 and HHV-7. The gammaherpesviruses have restricted host range *in vitro* and can replicate in different cell types, including lymphocytes and endothelial cells. In lymphocytes, the major latent reservoir, these viruses can be re-activated from latency. The gammaherpesviruses include HHV-4 (Epstein-Barr virus, EBV) and HHV- 8.

α	HHV-1	Herpes-simplex virus (HSV-1)	Oropharyangeal herpes Genital herpes
α	HHV-2	Herpes-simplex virus (HSV-2)	Genital herpes
α	HHV-3	Varicella-Zoster virus (VZV)	Varicella Zoster
β	HHV-5	Human cytomegalovirus (HCMV)	CMV-mononucleosis CMV disease
β	HHV-6	Human herpesvirus 6	Exanthem subitum
β	HHV-7	Human herpesvirus 7	Exanthem subitum
γ	HHV-4	Epstein-Barr virus (EBV)	Infectious mononucleosis Nasopharyngeal carcinoma Burkitt's lymphoma Classical Hodgkin's lymphoma
γ	HHV-8	Kaposi's sarcoma-associated herpesvirus (KSHV)	Kaposi's sarcoma Primary effusion lymphoma Multicentric Castleman's disease

Table 1.1. Human herpesviruses diseases.

1.1.1 Virion morphology

The virion of HCMV consists of a linear DNA genome, which is contained within an icosahedral capsid of about 100-110nm in size. The capsid is composed of 162 pentameric and hexagonal capsomere subunits and appears similar to the HSV-1 capsid. This, in turn, is surrounded by an amorphous tegument layer and a membranous envelope, the latter of which carries a large number of glycoproteins. The complete virion, consisting of the capsid core, the tegument and the envelope is about 150-200nm in size, reflecting differences in the size of the tegument (Mocarski and Courcelle, 1996). Figure 1.1 is a schematic representation of the CMV virion structure. CMV infection of cultured cells results in the production of two additional extracellular forms of virus particles: dense bodies and non-infectious virus particles. Dense bodies are composed of the tegument and viral envelope only, while the non-infectious virus particles lack the DNA core.

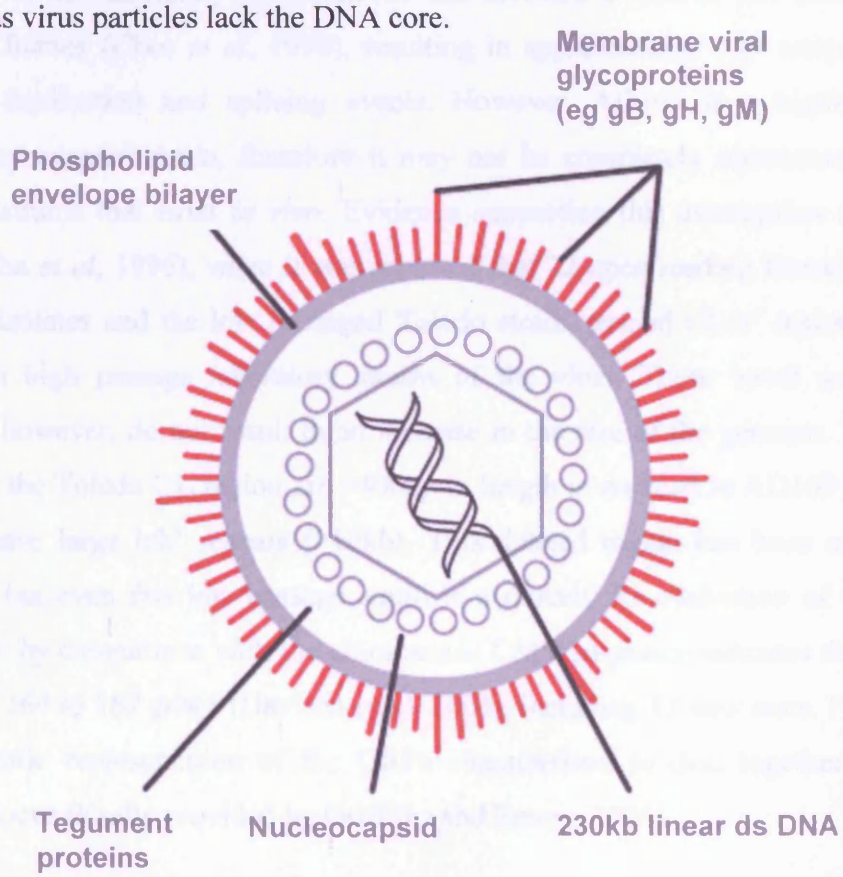


Figure 1.1. Morphology of the HCMV virion. The capsid containing the linear DNA genome is surrounded by the tegument layer and the envelope.

1.1.2 Genomic arrangement

The human CMV genome has more sequence complexity than most other herpesviruses. It is the largest herpesvirus genome consisting of linear DNA, 230kb long. CMV is also the only betaherpesvirus known to contain a class E genome structure, comprising a genome with direct and inverted repeat elements, which can undergo inversion and result in four sequence isomers. The genome is divide into two unique components, the unique short region (US) and the unique long region (UL). Inversion of these regions is mediated by repeated sequences at the genomic termini and the UL-US junction. The inverted repeats b (TR_L/IR_L) and c (IR_S/TR_S) flank the unique regions of UL and US. A directly repeated α sequence is found at the genomic termini and is also present in inverted orientation at the UL-US junction. Sequence analysis of the laboratory strain AD169 has revealed a total of 208 predicted open reading frames (Chee *et al*, 1990), resulting in approximately 189 unique proteins, due to duplication and splicing events. However, AD169 is a highly passaged laboratory adapted strain, therefore it may not be completely representative of the HCMV strains that exist *in vivo*. Evidence supporting this assumption appeared in 1996 (Cha *et al*, 1996), when it was reported that 22 open reading frames present in clinical isolates and the low passaged Toledo strain, termed UL/b' region, were not found in high passage laboratory strains of the virus. These novel sequences of Toledo, however, do not result in an increase in the size of the genome. The repeats flanking the Toledo UL region are ~400bp in length in contrast to AD169 and Towne which have large b/b' repeats (>10kb). This deleted region has been examined in Toledo, but even this low passage strain is a mutant. Re-evaluation of the HCMV sequence by comparison with the chimpanzee CMV sequence indicates that the virus encodes 164 to 167 genes (Davison *et al*, 2003), including 11 new ones. Figure 1.2 is a schematic representation of the ORFs characterised to date together with their genetic locus (kindly provided by Griffiths and Emery, 2002).

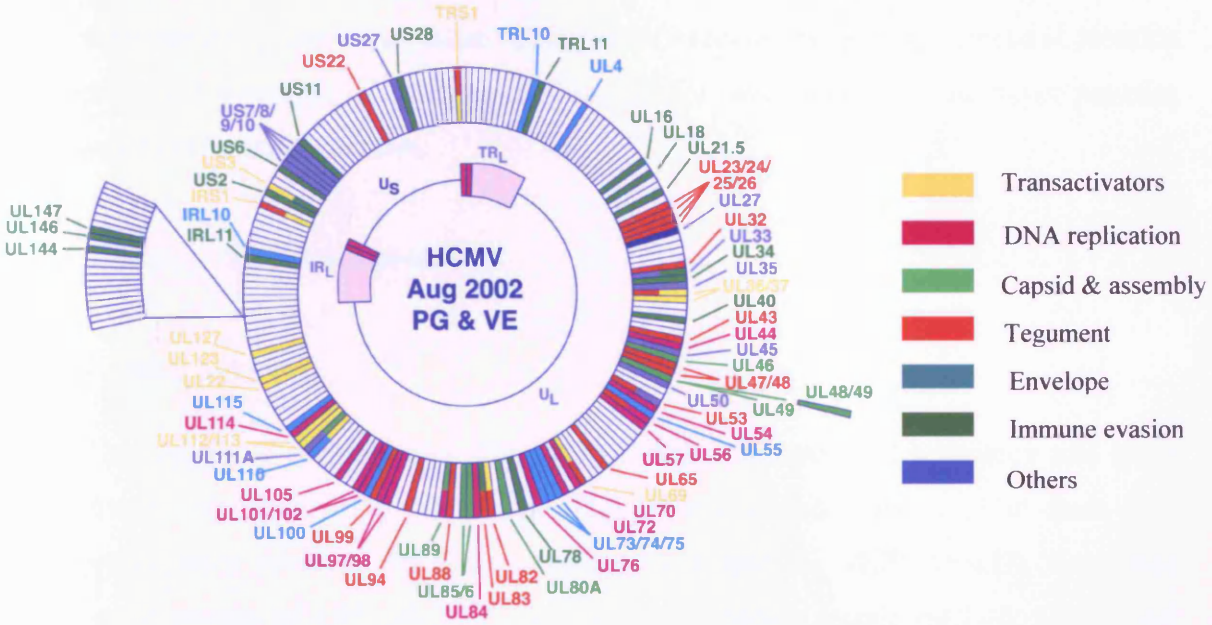


Figure 1.2. Localisation of genes encoding HCMV proteins (kindly provided by Griffiths and Emery, 2002).

1.1.3 Proteins

Since the sequencing of the HCMV genome, the protein nomenclature has been standardised such that a protein mapped to an open reading frame on the viral genome should be given a name that corresponds to the open reading frame, preceeded by a prefix. The prefix gp denotes glycoprotein, pp phosphoprotein and p where the characteristics of the protein are unknown. HCMV proteins are generally grouped into the structural proteins that make up the virion structure and the non-structural proteins that are responsible for viral replication. A brief description of some major proteins and their functions follows.

1.1.3.1 Structural proteins

Capsid proteins

The capsids of herpesviruses show a high degree of structural homology and share closely related assembly pathways. The HCMV capsid consists of at least four predominant proteins: the major capsid protein (pUL86, MCP, 153kD), the minor capsid protein (pUL85, 34kD), the minor capsid binding protein (pUL46, 33kD), and the smallest capsid protein (pUL48/49, SCP, 8.5kD) (Irmiere and Gibson, 1985, Gibson *et al*, 1996). The major capsid protein shows low imunogenicity in humans and a high degree of homology to the VP5 protein of HSV (Jahn and Mach, 1990), the smallest capsid protein is essential for the assembly of infectious virus in HCMV-infected cells (Borst *et al*, 2001). It has been recently documented that the MCP and SCP interact in the cytoplasm of transfected cells through two linear sequences in the SCP (Lai and Britt, 2003).

Tegument proteins

The tegument is situated between the capsid and the envelope and comprises mostly phosphoproteins. Although it occupies a large part of the HCMV virion (~40% total mass) (Irmiere and Gibson, 1983), there is little knowledge about its function. Comparison with other herpesviruses predicts that it may be essential for viral gene regulation and envelopment. The most abundant tegument proteins are pp65 (ppUL83

or lower matrix protein) and pp150 (ppUL32 or basic phosphoprotein), both able to produce a dominant immunologic response in humans. pp65 can be detected HCMV-infected cells in culture within 24 hours post-infection (Revello *et al*, 1992) and is also present in the peripheral blood of immunocompromised patients with active HCMV infection (Grefte *et al*, 1995). This protein constitutes a major target for the cytotoxic T cell immune (CTL) response in humans (He *et al*, 1995, Akiyama *et al*, 2002, Allart *et al*, 2003). Other tegument proteins include pp71 (ppUL82 or upper matrix protein), pp28 (UL99) and pp130 (UL56). The upper matrix protein is highly homologous to pp65 but less abundant. It is a transactivator of gene expression along with pUL69 (Ishov *et al*, 2002). It facilitates viral genome deposition and transcription at nuclear domains 10 (ND10), possibly preparing the virus for more efficient productive infection. pp28 is highly immunogenic and pp130 is involved in virion maturation.

Envelope proteins

The HCMV envelope contains viral proteins and cellular lipids that are derived from either the nuclear or cytoplasmic membrane. Sequence analysis has shown that many of the viral proteins (~55) are glycoproteins (Chee *et al*, 1990). Of these, only two have been characterised CMV glycoprotein B (gB or gpUL55) and glycoprotein H (gH or gpUL75). gB is the most abundant glycoprotein and shares sequence homology with related proteins in HSV, VZV, HHV-6 and HHV-7. It is a transmembrane protein, containing two components, which are linked together by disulphide bonds. It is the major target for neutralising antibody responses in humans and is also the target for $CD4^+$ and $CD8^+$ T cell responses, making it a good candidate for use in subunit vaccines (Britt and Vugler, 1990, Riddell *et al*, 1991, Temperton *et al*, 2002). Glycoprotein B has been implicated in cell penetration, fusion of infected cells, cell to cell transmission and the alteration of cellular transcription early during infection (Tugizov *et al*, 1994, Simmen *et al*, 2001). In epithelial cells, the cytosolic domain of gB is responsible for internalisation from the cell surface into clathrin-coated vesicles, mediating HCMV entry in the endocytic pathway (Tugizov *et al*, 1999).

Glycoprotein H is less abundant than gB but has been shown to elicit ~30% of the total neutralising activity in human sera. It was reported in 1994 that antibodies to gH in AIDS patients were essential for limiting retinitis (Rasmussen *et al*, 1994). Some other glycoproteins that have been mapped to viral open reading frames include gL (gpUL115), gO and the gcII complex. These proteins along with gH have been shown to play roles in virion attachment to the cell, fusion and penetration (Kari and Gehrz, 1992, 1993). Finally, the gM glycoprotein associates with gN to form a complex that induces a neutralising antibody response in the host (Mach *et al*, 2000, Pignatelli *et al*, 2001).

1.1.3.2 Homologues of cellular proteins

HCMV encodes a number of proteins that are homologous to cellular proteins. UL33, UL78, US27 and US28 are homologous to the G-coupled receptor proteins (Rawlinson *et al*, 1996) and are involved in the interaction between the virus and a variety of different cell types during infection. US28 encodes a C-C chemokine receptor that binds RANTES and the monocyte chemoattractant protein 1. This binding increases calcium levels in the cells and provides a favourable environment for viral gene expression (Neote *et al*, 1993). The HCMV UL146 and UL147 ORFs encode α chemokine homologues (Penfold *et al*, 1999), suggesting they may interfere with neutrophil activity during HCMV. Additionally, UL144 has been shown to be homologous to members of the tumour necrosis factor receptor (TNFR) superfamily (Benedict *et al*, 1999, Lurain *et al*, 1999). It was found that the UL144 protein was expressed early after infection and was retained in an intracellular compartment. This gene product is important because of its possible role in HCMV pathogenesis as a growth factor decoy that facilitates evasion of the immune response by the virus. HCMV also encodes MHC class I homologues, UL18 and UL142 (Beck and Barrel, 1988, Tomasec, 2000, Wills *et al*, unpublished data). The expression of these genes may contribute to immune evasion. HCMV also contains a gene, which is homologous to the R1 subunit of ribonucleotide reductase (RNR). RNR is a cellular gene with an essential role in deoxyribonucleotide synthesis, consisting of an R1 and an R2 subunit. The host enzyme is expressed in proliferating cells. M45 protein of murine CMV exhibits sequence homology to R1 RNR, but lacks some amino acids

believed to be critical for enzymatic function. Experiments using a polyclonal antibody against a recombinant M45 protein showed that the protein was expressed at 12 hours after infection of mouse fibroblasts, was associated with the virion particle and was not a functional equivalent of a RNR R1 subunit. Mutant viruses containing an inactivated M45 gene displayed a reduced growth *in vitro*, failed to replicate in organs of Balb/c mice and did not cause any mortality. These data suggest that M45 is indispensable for virus replication *in vivo* (Lembo *et al*, unpublished data). Some herpesviruses encode proteins with FcγR activity (Costa *et al*, 1978, Baucke *et al*, 1979). In murine CMV an FcγR protein is encoded by the early gene m138 (Thale *et al*, 1994), which has no homologue in the HCMV AD169 sequence. The deletion of this gene resulted in an attenuated phenotype *in vivo* (Crnkovic-Mertens *et al*, 1998). Recently, however, two glycoproteins in AD169, named UL119-118 and gpTRL11, were found to be homologous to and share functional properties with FcγR (Atalay *et al*, 2002). Lastly, homology between the HCMV UL1 protein and the CEA/PSG human protein family has been documented (Holzerlandt *et al*, 2002). CEA family members are involved in cell adhesion, signal transduction and innate immunity (Hammarstrom, 1999). PSGs, a CEA family subgroup, are expressed in the placenta and possibly regulate immune system responses. The homology between UL1 and PSGs could contribute to the pathology of HCMV during pregnancy.

1.2 Entry of HCMV into host cells

One of the factors required for successful viral replication is the ability to gain access into a susceptible cell. CMV infection begins by the low affinity attachment of the virus on the cell, followed by high affinity attachment, fusion of the viral envelope with the cell membrane, uncoating of the nucleic acid and transport of the viral DNA to the cell nucleus. The initial low affinity attachment is mediated by an interaction between cell surface heparan sulphate (Compton *et al*, 1993) and viral glycoproteins, most probably gB and gcII complex (Kari *et al*, 1992, 1993) and followed by high affinity binding, which is heparin resistant, which may be regulated by CD13, an aminopeptidase (Soderberg *et al*, 1993). Studies have shown that CMV infection and binding of virions to cells, can be inhibited by antibodies directed against CD13. The viral ligand involved in this high affinity binding is not known yet.

Findings suggest that annexin II, a 30kD member of the lipocortin family, is the principal CMV receptor involved in virus binding to cells and cell to cell spread (Wright *et al*, 1994, Bold *et al*, 1996). However, another group using a different cell type, demonstrated that neutralisation of cell surface annexin II was inconsequential for direct cell to cell spread of the virus (Pietropaolo and Compton, 1999). It is therefore possible that the role of annexin II in HCMV entry, as well as the entry mechanism itself, differs from cell type to cell type. MHC (major histocompatibility complex) class I molecules have also been suggested as possible receptors for HCMV. However, none of the interactions of the virus with these receptors has been linked to HCMV-induced signalling and entry. Only recently, it was revealed that the EGFR (epidermal growth factor receptor) is a necessary receptor for HCMV (Wang *et al*, 2003). Wang *et al* showed that HCMV infects cells through interaction of viral gB with EGFR, triggering an intracellular signalling cascade.

Following the attachment of HCMV to the cell surface, virus penetration occurs by pH independent fusion of the viral envelope with the cell membrane. It has been demonstrated that gB and gH are essential in this process. Antibodies against gB have been shown to interfere with HCMV penetration and cell to cell spread of infection (Navarro *et al*, 1993), while similar observations were made with antibodies directed against gH. Entry of HCMV in endothelial and retinal pigment epithelial cells occurs through endocytosis (Bodaghi *et al*, 1999). Finally, the nucleocapsid is released into the cytoplasm, allowing the launch of virus replication after entry in the nucleus.

1.3 HCMV replication

After the nucleocapsid is released into the cell cytoplasm it enters the nucleus, capsid proteins are removed followed by DNA genome circularisation and the onset of replication. Differential gene expression during viral replication plays a crucial role in determining whether the resulting infection will be productive, abortive or latent. Therefore, the extent of genome expression in any given cell type shapes the pattern of the virus-host interaction.

The expression of HCMV genes during productive replication is temporally regulated. The full replication cycle of HCMV *in vitro* takes approximately three days, however

it has been estimated from studies *in vivo* that this does not reflect the replication time in host cells *in vivo*. Through such modelling studies the *in vivo* doubling time of HCMV can be assessed as one day (Emery *et al,* 1999, 2002). During HCMV lytic replication viral genes are expressed sequentially in three phases, termed immediate early (IE or α), early (E or β) and late (L or γ), according to the timing of transcription (Table 1.2).

Class	**Time of expression after infection (hr)**	**Definition**
Immediate-early , α	1	Expression requires no prior viral protein synthesis.
Early , β1	4–8	Expression is independent of virus DNA synthesis.
Delayed early , β2	8–24	
Late , γ	48–72	Expression requires virus DNA synthesis.

Table 1.2. Regulatory classes of herpesvirus genes expressed during lytic replication after infection. α, β and γ in this case, refer to the gene expression classes rather than the viral subfamily.

The first genes (immediate early, IE genes) are expressed within an hour of infection and their expression is independent of any viral *de novo* protein synthesis. The expression of the IE genes is confined to discrete regions of the viral genome, although the main site of IE transcription is in the UL component (Mocarski and Courcelle, 1996). Their products are non-structural proteins, essential for switching on early gene expression. The IE 1 and 2 proteins are known transcription factors, which regulate the expression of viral and cellular genes (Caswell *et al*, 1996). Several studies have uncovered similarities between the functions of HCMV IE proteins and IE equivalents encoded by adenovirus, such as the targeting of retinoblastoma and p53 families and the induction of S-phase in resting cells. Despite this, HCMV encodes several other IE proteins that act early in infection to prevent host DNA replication and apoptosis (Castillo *et al*, 2002).

The next set of genes (early, E genes) are expressed in two phases, name early or β1, with transcription occurring at 4-8 hours after infection, and delayed early β2, with transcription occurring at 8-24 hours after infection. They encode products that are involved in DNA replication, DNA repair, and other non-structural proteins. Viral DNA synthesis is initiated from the origin of replication (ori_{lyt}) and proceeds in both directions to form a rolling circle, yielding "head to tail" concatameric DNA.

The last set of genes, (late, L genes) are expressed after the onset of viral DNA replication, at about 48-72 hours post infection *in vitro*. These encode mainly structural proteins such as capsid, tegument and envelope proteins. Additionally, some of these proteins may play an important role in regulating transcription, viral assembly, as well as altering host cell proteins. The expression of HCMV proteins can be inhibited at several stages with the use of metabolic inhibitors, as illustrated in Figure 1.3.

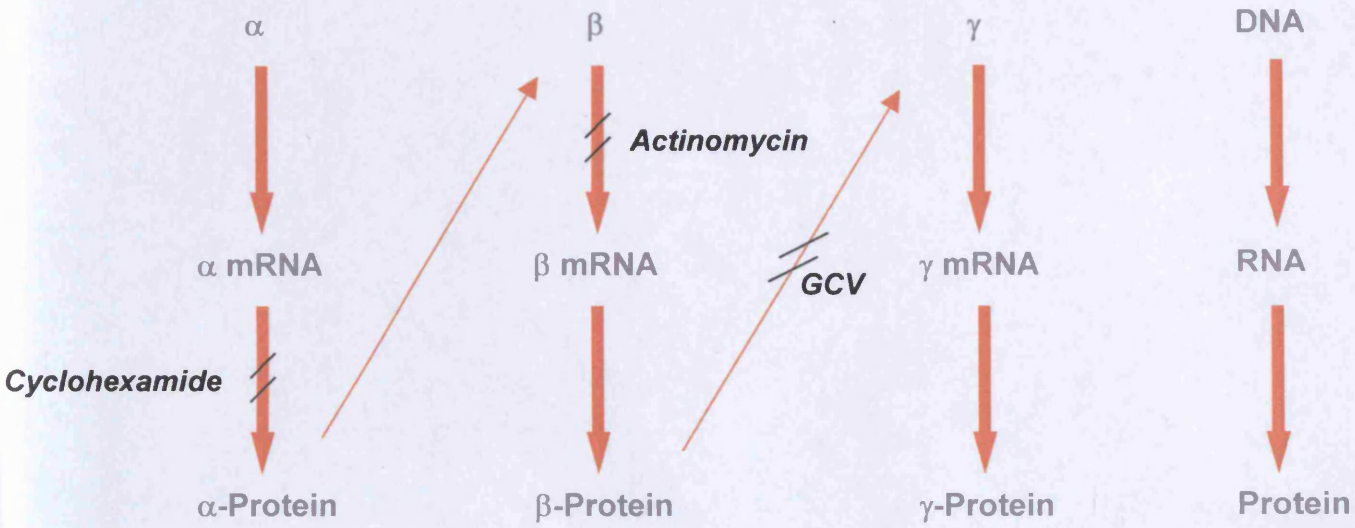


Figure 1.3. HCMV gene expression. This diagram also shows the stages at which metabolic inhibitors can be employed to manipulate the cascade. Cyclohexamide is an early protein synthesis inhibitor; actinomycin is a transcriptional inhibitor; GCV is a late protein synthesis inhibitor. GCV=ganciclovir.

During the synthesis of virion components and late DNA replication, virus particles begin to assemble in the nucleus. DNA is packaged into the mature capsid, followed by the assembly of tegument, which is thought to occur when the nucleocapsids bud through the nuclear membrane, where initial envelopment may take place. It has been recently reported that UL97 is required for nucleocapsid nuclear egress (Krosky *et al*, 2003). Although it is still unclear whether the final virus envelope is derived from the nuclear or the cytoplamic cell membrane, it has been shown recently that the final envelopment of HCMV particles takes place mainly in a Golgi-derived secretory vacuole (Homman-Loudiyi *et al*, 2003), which may act to release new infectious particles by fusion with the plasma membrane. Viral glycoprotein insertion and virus egress of the mature virion are thought to occur in the endosomal compartment of the cell (Tooze *et al*, 1993). Once produced, the virus can be released to the extracellular body fluid or may infect neighbouring cells via cell-to-cell spread. Figure 1.4 illustrates the different steps in the life cycle of HCMV.

1.4 The strains of HCMV and identification of the UL/b' region

AD169, Towne and Toledo are HCMV strains commonly used in the laboratory environment. Clinical data have demonstrated that HCMV strains exhibit different levels of virulence depending on their passage history in cell culture. AD169 and Towne were developed as potential live, attenuated vaccine candidates by serial passage of a clinical isolate in fibroblast cultures. AD169 was passaged over 50 times in fibroblasts after being isolated from the adenoids of a 7-year-old girl undergoing tonsillectomy-adenoidectomy (Elek and Stern, 1974). Towne was isolated from the urine of a congenitally infected 2-month-old infant and was passaged over 128 times in fibroblasts (Plotkin *et al*, 1984). Both strains were tested in controlled human trials and shown to be avirulent in HCMV seronegative volunteers. In contrast, a low passaged strain, Toledo, has produced clinical disease when administered to healthy adult volunteers (Quinnan *et al*, 1984). Toledo was derived as a challenge virus during development of the Towne vaccine and was passaged for only five times on fibroblasts. It has also been shown that Toledo is more virulent that both AD169 and Towne when inoculated into SCID-hu (thymus plus liver) mice (Brown *et al*, 1995). It has been speculated that Toledo contains genes important for tropism/replication

efficiency, which have been lost by other strains upon extensive passage in fibroblast cell culture.

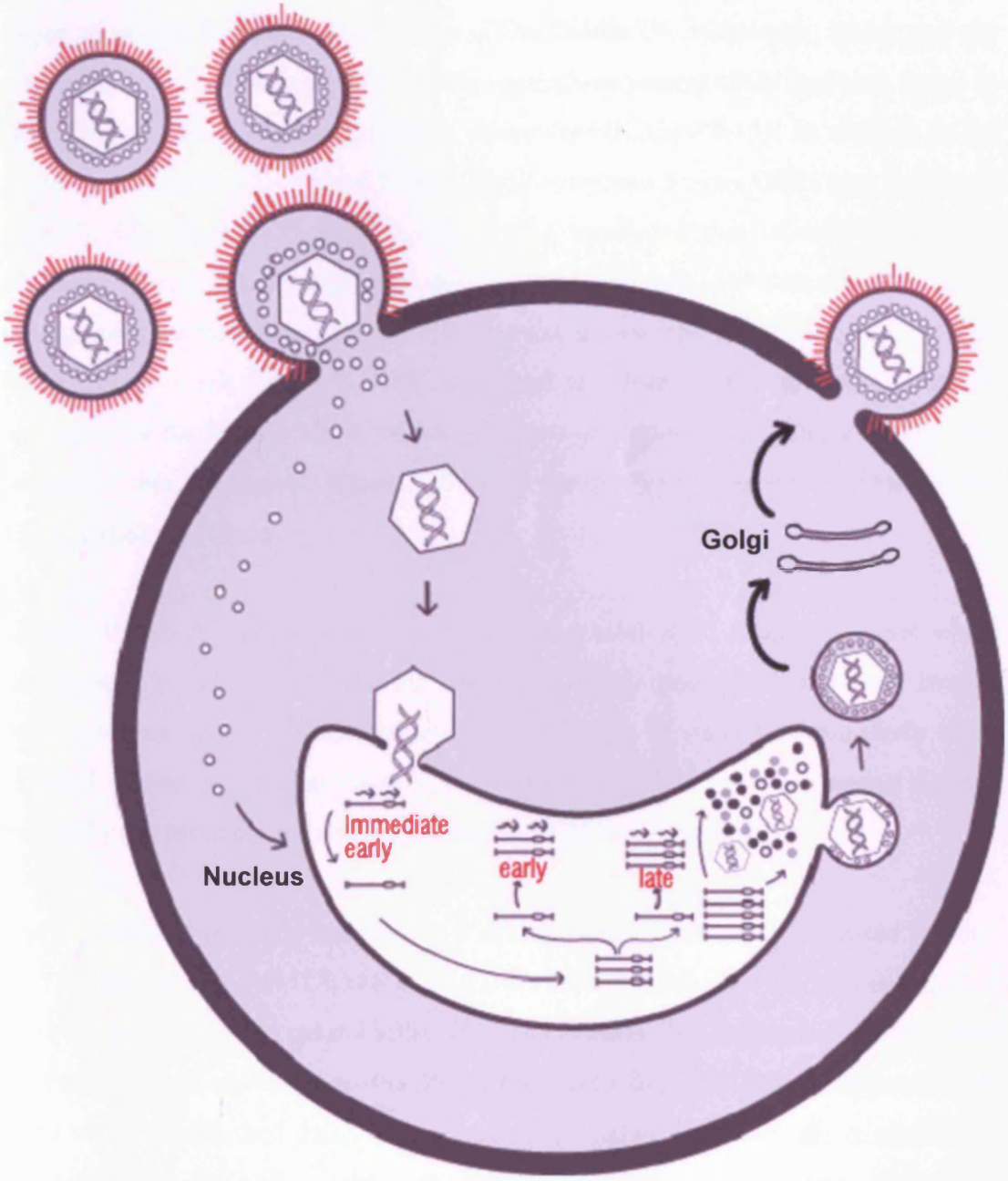


Figure 1.4. A schematic representation of the life cycle of HCMV. After virus adsorption, the nucleocapsid is released into the cell cytoplasm and enters the nucleus. Capsid proteins are removed followed by the onset of replication. During HCMV lytic replication viral genes are expressed sequentially in three phases (IE, E, L). At the late stage of DNA replication, virus particles begin to assemble in the nucleus, DNA is packaged into the mature capsid, followed by the assembly of tegument and nucleocapsid budding through the nuclear membrane. Once produced, the virus can be released to the extracellular body fluid or may infect neighbouring cells via cell-to-cell spread.

An extensive comparison of the restriction enzyme profiles of Toledo and a highly passaged variant of Towne combined with genome sequencing revealed an additional region of unique DNA at the right edge of the Toledo UL component, which was not present in AD169 (Cha *et al*, 1996). This region was termed UL/b' and was found to span 13kb encoding 19 potential ORFs, designated UL133-UL151. In addition to the 19 genes missing in AD169 and Towne, Towne contains 3 extra ORFs (not present in AD169): UL152, UL153 and UL154. It was concluded that AD169 and Towne contained segments homologous to the UL/b' region that were lost upon extensive passage of these viruses in culture. Hybridisation showed that a portion of the variants in the Towne stock from the ATCC contained sequences highly homologous to the sequences of the Toledo UL/b' region (Duke *et al*, unpublished data), a finding that supported this conclusion. Figure 1.5 shows the predicted genome structure in the UL/b' region of Toledo.

Overall, the UL/b' region is thought to contain a total of 22 additional genes which are present in clinical isolates but absent in highly passaged laboratory strains. Although these genes are not essential for replication *in vitro*, they are likely to be essential *in vivo*. The maintenance of these ORFs in clinical isolates suggests that the encoded gene products are also important for HCMV pathogenesis *in vivo*.

The functions of products encoded by 5 of these genes has been investigated to date: UL141, UL142, UL144, UL146 and UL147. Recent studies on UL141 and UL142 have suggested a role in natural killer (NK) cell evasion. The expression of both genes in fibroblasts was shown to protect the infected cells from NK lysis (Wilkinson *et al*, Wills *et al*, unpublished data). The UL146 and UL147 ORFs encode α chemokine homologues (Penfold *et al*, 1999). The UL146 gene from clinical isolates from AIDS patients is one of the most variable genes within the entire HCMV genome. The nucleotide sequence variability is less than 70% for UL146, resulting in a high level of variation at the amino acid level. The primary amino acid sequence from a clinical isolate was only 37% identical to Toledo UL146, resulting into different functional outcomes in clinical isolates (Sparer *et al*, unpublished data). This sequence divergence is highly unusual, since there is a >95% nucleotide sequence conservation for the rest of the HCMV genome. The expression of UL146 has been documented in

many clinical isolates. *In vitro* experiments using a recombinant virus showed that this gene product is present in cells lysates, with a heavily glycosylated form secreted in the media. Function studies indicated that the protein could induce calcium mobilisation and neutrophil chemotaxis. The nucleotide and amino acid sequence of UL147 are also highly variable within clinical isolates. UL146 and UL147 may interfere with neutrophil activity during HCMV infection and consequently the loss of the UL/b' region could contribute to the attenuation of AD169.

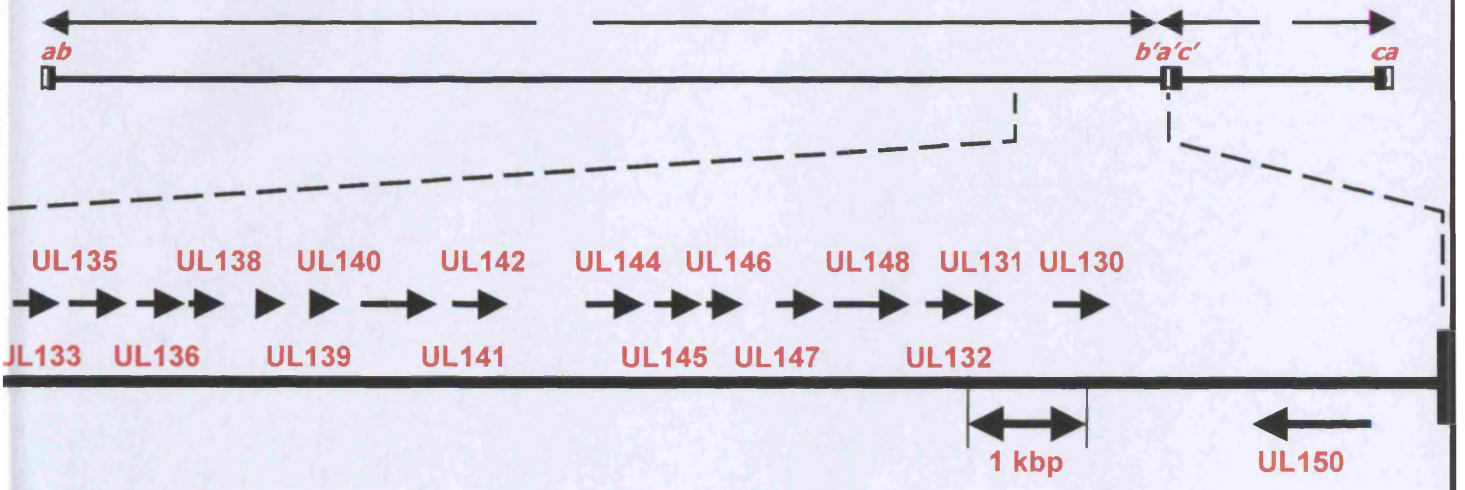


Figure 1.5. Illustration showing the layout of genes at the right end of UL in Toledo (Figure taken from Davison *et al*, 2003).

UL144 is conserved in more than 45 low passage clinical isolates (Lurain *et al*, 1999), but shows sequence hypervariability. Therefore, clinical isolates cluster into three major groups, based on UL144 sequence. Looking at UL144 sequence in isolates derived from the same person illustrated that UL144 remains unchanged within an individual. The predicted amino acid sequence contains elements of a type I transmembrane protein and UL144 was shown to be homologous to HVEM (herpesvirus entry mediator), and other members of the tumour necrosis factor receptor (TNFR) superfamily (Benedict *et al*, 1999, Lurain *et al*, 1999). HVEM is required for HSV-1 entry into T cells (Mauri *et al*, 1998). UL144 protein is expressed early after infection and retained in an intracellular compartment, showing no cell surface expression (Benedict *et al*, 1999). Furthermore, it does not interact with any of the known TNF-related ligands, suggesting that this protein lacks a functional ligand-binding domain. UL144 is important because of its possible role in HCMV pathogenesis as a growth factor decoy that facilitates evasion of the immune response by the virus.

Additional differences between the low and high passaged HCMV strains exist outside the UL/b' region. Different variants of AD169 have a 929bp deletion near UL42-UL43, compared to Toledo (Dargan *et al*, 1997, Mocarski *et al*, 1997). The coding region of UL36 gene of Towne has a deletion of several hundred nucleotides relative to the one in Toledo (Goldmacher *et al*, unpublished data). Additionally, the origin of lytic phase replication in Towne contains repeat sequences, which are missing from Toledo viral DNA (Anders *et al*, 1992, Masse *et al*, 1992, Kemble *et al*, 1996).

HCMV replication in a particular cell type is also dependent on the virus strain used. *In vitro* studies have shown that endothelial cells are not generally permissive for infection with high passage strains of HCMV (Friedman *et al*, 1981). However, these cells are permissive for infection with some low passage HCMV isolates (Ho *et al*, 1984, Waldman *et al*, 1989). Monocyte-derived immature dendritic cells were shown to only support the full replication cycle of endothelial cell tropic strains of HCMV *in vitro*, resulting in the expression of IE, E and L viral genes and production of infectious virus (Riegler *et al*, 2000). It has been suggested that low passage strains of HCMV contain a number of endothelial cell tropic variants within a mixed population

of strains that are lost after extended passage in fibroblasts, rather than deletion/mutation of existing genomes accounting for the observed effects (MacCormac and Grundy, 1999).

1.5 Cell tropism of HCMV and determinants of replication

Although HCMV receptors are found on a variety of cell types *in vitro*, entry of virus does not always result in the establishment of permissive infection in culture. The extent of HCMV infection ranges from lytic infection, to low-level persistent infection, to abortive infection, and finally, to latency, suggesting that host cell factors play a significant role in controlling replication of the virus.

Blood monocytes appear to be abortively *in vitro*. However, they become permissive once they have differentiated to macrophages following phorbol ester treatment (Weinshenker *et al*, 1988). HCMV replication can also be stimulated in some endothelial and epithelial cells after they have been treated with factors that promote cellular differentiation, such as sodium butyrate (Tanaka *et al*, 1991, Wu *et al*, 1994). In addition, the induction of cell differentiation by retinoic acid in teratocarcinoma cells, which are otherwise non-permissive for HCMV infection, promotes productive infection (Gonczol *et al*, 1984).

Most of the above *in vitro* studies have been performed using strains of HCMV that have been passaged in fibroblasts many times, which can affect the tropism for other cell types. The importance of the infecting strain of HCMV for clinical outcome has been the subject of speculation since it was first recognised that all epidemiologically unrelated clinical isolates have a unique genetic profile (Huang *et al*, 1976). Although they have a high level of sequence homology, they can be readily differentiated by restriction enzyme analysis of full-length genomic DNA and immunologic assays (Waner *et al*, 1978). Different HCMV strains also display biological differences, the genetic basis for which is still being investigated. Studies have shown that low passage clinical isolates can readily infect human endothelial cells and polymorphonuclear leukocytes isolated from human blood (Waldman *et al*, 1991, Grundy *et al*, 1998, Sinzger *et al*, 1999). Additionally, while the AD169 strain was found to infect fibroblasts efficiently, it showed a low infectivity profile to smooth

muscle cells, whereas a low passage clinical isolate exhibited comparable infectivity characteristics on both cell lines (Woodroffe *et al*, 1997). These tropism differences between high and low passage strains suggest that different tissue tropism may occur *in vivo*. Indeed, differences in the distribution of CMV infection in leukocytes from tissues of patients have been demonstrated (Sinzger *et al*, 1996). Another study showed that different clinical isolates obtained from transplant patients had specific tropism for different cells (Torok-Storb *et al*, 1993) and therefore it was proposed that the variation in tropism might account for differences in pathogenicity. The association between different gB genotypes and different tissue distribution of the virus in subsequent studies strengthen this statement (Rasmussen *et al*, 1997, Meyer-Konig, 1998). Variability in gene sequence of low passage isolates has also been reported for UL11 (Hitomi *et al*, 1997) and exon 3 of the IE gene (Hebart *et al*, 1997). However, there is contradictory data on the association between gB genotypes and clinical outcome. Several studies have shown no correlation was between the gB genotype and the development of CMV disease following liver or kidney transplantation (Sarcinella *et al*, 2002, Pacsa *et al*, 2003).

HCMV Towne lacks two biological properties that are present in clinical isolates: endothelial cell tropism and polymorphonuclear leukocyte tropism. It has been shown however, that Towne can reacquire tropism for both cell types after its adaptation to growth in endothelial cells. Leukotropism was reacquired 10-20 passages later than reversion to endothelial cell tropism, implying that these two biological activities are distinct (Gerna *et al*, 2002). Furthermore, AD169 was also shown to reacquire both properties *in vitro* (Gerna *et al*, 2003). Recently, the genome region that determines leukocyte and endothelial cell tropism has been mapped to the UL131-UL128 locus. Transcript mapping data showed that UL128 and UL131A genes are spliced, and they flank UL130, which is unspliced (Davison *et al*, 2003). All three genes are expressed at late times after infection. Sequence analysis suggests that UL128 encodes a β chemokine (Akter *et al*, 2003). Compared with two HCMV isolates analysed directly from infected human tissues, each of eight isolates that were passaged in fibroblasts contained a mutation in one of these genes. Additionally, AD169 had a frameshift in UL131A and Towne in UL130. In Toledo, UL128 is disrupted by an inversion of part of the genome. An alternative approach by Hahn *et al* used a wild type virus strain

with endothelial and leukocyte cell tropism and cloned it as a bacterial artificial chromosome (BAC) in *E.Coli* (Hahn *et al*, unpublished data). Virus mutants with a deletion in either individual genes UL128, UL130, UL131 or the entire UL131-UL128 region lost HCMV tropism for both endothelial cells and leukocytes. In contrast, deletion of the UL/b' region did not alter tropism. In addition, leukocyte but not endothelial cell tropism could be restored in knock-out mutants by trans-complementation in fibroblasts expressing the UL131-UL128 locus. The UL131-UL128 region is thought to encode 5 proteins with chemokine homology, namely UL131, UL131x1, UL128x1, UL128 and UL130. Baldanti *et al* also showed that loss of tropism for endothelial cells is associated with mutations in this region, by sequencing UL131-UL128 in tropism deficient HCMV variants and then adapted them to grow in endothelial cells, which led to recovery of endothelial and leukocyte cell tropism, along with a back-mutation in the UL131-UL128 region. However, again, only leukocyte tropism could be complemented in trans in fibroblasts expressing the wild type UL131-UL128. These results indicate that the UL131-UL128 products are sufficient for endothelial cell spread, as well as for the attraction of monocytes and subsequent viral transfer. However, additional chemotactic factors appear to be required for full expression of leukocyte tropism. The combined data from studies on the UL131-UL128 region suggest that it is intimately involved in determining tropism.

HCMV replication is also regulated at the level of viral entry. It has been shown recently that while HCMV enters both fibroblast and endothelial cells within five minutes of contact, entry into endothelial cells occurs via endocytosis, while entry into fibroblasts occurs via fusion (Bodaghi *et al*, 1999). Therefore, the sequestration and degradation of incoming particles in endocytic vacuoles in endothelial cells could account for the low level of infection seen in this cell type. It has also been shown that AD169 appears to be defective in its ability to translocate viral nucleic acid to the nucleus of human umbilical vein endothelial cells (HUVEC) following entry of the virus into the cell (Slobbe-van Drunen *et al*, 1998, Sinzger *et al*, 2000).

Histopathological analyses of autopsy material from HCMV-infected individuals have demonstrated the presence of HCMV in virtually all the organs of the body. The major sites of viral replication include the salivary glands, liver, kidney, brain, heart,

eye, GI tract, placenta and peripheral blood (Egbert *et al*, 1980, Wreghitt *et al*, 1988, Schmidt *et al*, 1993, Sinzger *et al*, 1995). Fibroblasts seem to be the major cell type infected in the lungs, intestine and the placenta (Sinzger *et al*, 1993). This is reassuring, since it provides a degree of relevance for the *in vitro* studies in fibroblasts. Apart from fibroblasts, the virus replicates in many different cells within the infected host, such as macrophages, smooth muscle, neurons, trophoblasts, hepatocytes, epithelial and endothelial cells (Ibanez *et al*, 1991, Sinzger *et al*, 1996, Gabrielli *et al*, 2001).

It is thought that haematopoietic cells and their progenitors play important roles in HCMV latency and replication (Hahn *et al*, 1998), with evidence suggesting that monocytes are the key site of latency *in vivo* (Soderberg–Naucler *et al*, 1997). During active HCMV infection, viral DNA can be detected in monocytes, polymorphonuclear cells (PMNLs), B cells and T cells of immunocompromised patients (Gerna *et al*, 1992, Boivin *et al*, 1998). Although PMNLs contribute the greatest to the overall viral load in blood ($\sim 10^5$ genomes per ml of blood), monocytes have the highest viral burden than any other leukocyte population (1 genome per 2 cells) (Hassan-Walker *et al*, 2001), illustrating the importance of this cell type the carriage of HCMV during viremic episodes. Detection of HCMV transcripts in leukocytes *in vivo* though, has produced conflicting results. In the bloodstream of healthy carriers, viral gene expression in monocytes is limited to immediate early genes (Dankner *et al*, 1990, Taylor-Wiedeman *et al*, 1991, Larsson *et al*, 1998). In contrast, the presence of IE and L viral transcripts, was demonstrated in all leukocyte populations by other groups (Von Laer *et al*, 1995, Hassan-Walker *et al*, 2001). It is widely accepted however, that tissue macrophages support full replication of the virus, with detection of early and late antigens (Sinzger *et al*, 1996). Additionally, it has been shown that HCMV can be transmitted by blood transfusion, where leukocyte depletion is associated with a reduction in the rate of HCMV transmission, indicating that infectious virus is predominantly cell associated. During latency the virus genome exists in a circular episomal conformation (Bolovan-Fritts *et al*, 1999). A model has been proposed where HCMV establishes a latent infection in monocytes, and is able to reactivate and generate a productive infection upon their differentiation into tissue macrophages. The reactivation of HCMV *in vivo* is a frequent phenomenon that occurs in both healthy carriers and immunosuppressed individuals (Soderberg-Naucler *et al*, 1997,

Prosch *et al*, 1999). HCMV is reactivated under conditions of allogeneic stimulation, for example after transplantation or blood transfusion. *In vitro* evidence has also suggested a role for the proinflammatory cytokine tumor necrosis alpha (TNF-α) in virus reactivation probably via the stimulation of IE genes (Hahn *et al*, 1998, Prosch *et al*, 1999).

1.6 Spread within the host

In vivo, the epithelial cells of the GI, genitourinary tract and respiratory tracts are the first to be infected by the virus. HCMV then gains access to the peripheral blood by an unknown mechanism and can further infect fibroblasts, epithelial cells and endothelial cells coating the blood vessels. The endothelial cells in turn, due to their crucial localisation at the interface between the organ tissues and peripheral blood, can pass the virus on to monocytes circulating in the blood. The virus replicates in these cells without causing cell lysis, by localising in endocytic vacuoles (Fish *et al*, 1995). This event makes monocytes a good vector for HCMV transfer to remote locations within the body.

During HCMV replication *in vivo*, the viral DNA is also detected in polymorphonuclear leukocytes (PMNLs), particularly neutrophils (Dankner *et al*, 1990). Additionally, the co-cultivation of neutrophils with endothelial cells infected with a low passage HCMV clinical isolate resulted in neutrophil acquisition of infectious virus (Grundy *et al*, 1998). Recently, HCMV was shown to encode a protein homologous to an α chemokine, capable of mediating neutrophil chemotaxis (Penfold *et al*, 1999). Therefore, apart from monocytes, endothelial cells could attract neutrophils to the site of infection and spread the virus, enabling viral dissemination within the body.

Finally, the infected endothelial cells could detach from the vessel wall, and enter the blood circulation. Indeed, the presence of infected cytomegalic cells in the blood of individuals with active HCMV infection was reported in 1995 (Sinzger *et al*, 1995). These circulating infected endothelial cells themselves could then transmit the virus to capillary endothelial cells and initiate organ infection.

1.7 Epidemiology

HCMV is distributed in human populations universally, with prevalence depending on socio-economic status and geographical location. It is acquired earlier in life in developing countries, infecting >90%, while infecting ~60% of individuals in the developed world (Wentworth and Alexander, 1971). Although in general HCMV infection rates are higher in nonwhites than whites, this variation probably reflects differences in socio-economic status rather than differences in racial orientation. Transmission can occur either vertically or horizontally.

HCMV can be spread from the mother to the baby either during gestation, birth or breastfeeding (Numazaki, 1997). The virus is the most common cause for congenital infections, infecting 0.25% to 2.5% of infants (Ahlfors *et al*, 1984, Griffiths *et al*, 1991, Murph *et al*, 1998). Infections of the fetus during gestation usually occur in women who were infected before conception as well as in those who have primary HCMV infection during pregnancy. Primary infection has a foetal transmission rate of 50% compared to less than 2% for recurrent infection. Additionally, it is quite rare for infants to develop clinically apparent HCMV disease when the mothers have pre-existing immunity to the virus (Fowler *et al*, 1992). Therefore, pre-existing immunity plays a role in the control of HCMV disease. The developmental stage of the fetus at the time of maternal primary infection is important, with infection occurring during the first trimester accounting for almost all the symptomatic cases (Stagno *et al*, 1986). Transmission of HCMV during birth depends on local shedding of the virus. Genital tract shedding is more common in women under 30 years of age. If the virus is being shed at the time of delivery, there is a 50% chance of transmission to the newborn (Reynolds *et al*, 1973). The most common route for transfer of the virus from the mother to the baby is during breastfeeding, with a strong relationship between the presence of viral DNA in milk and transmission (Vochem *et al*, 1998, Hamprecht *et al*, 2001). Although viral transmission during delivery and breastfeeding occurs more frequently than during gestation, it is not associated with the morbidity seen in congenital HCMV infection. However, asymptomatic infants shed the virus for a long period, contributing to the horizontal spread of HCMV in the community.

Although primary HCMV infection in the immunocompetent host is usually clinically silent, it can sometimes result in mononucleosis, which has similar clinical manifestations to EBV-induced mononucleosis (Pannuti *et al*, 1985). Some of the symptoms include fever, malaise, fatigue, elevated hepatic transaminases and pharyngitis. Following infection with HCMV, the virus can be detected in saliva, semen, tears and cervical secretions and remain latent in CD14 monocytes and other bone marrow progenitors. The horizontal transmission of HCMV requires direct contact with infectious material. The main routes of horizontal transmission are through sexual intercourse and person-to-person contact. Indeed, day care centres are major sites of risk for infection in pregnant women from children shedding virus in urine and saliva due to asymptomatic infection (Hutto *et al*, 1985, Pass *et al*, 1986, Shen *et al*, 1993, Kashiwagi *et al*, 2001).

HCMV can also be acquired via blood transfusion, although the use of screened blood products from seronegative donors has reduced the risk of infection (Bowden *et al*, 1986). This, however, limits the availability of donors. The use of procedures that remove the leukocytes (major cell type carrying HCMV in blood) from blood have been shown to decrease the risk of virus transmission. Finally, the transplantation of an organ from an infected individual can also result in HCMV transmission within the population.

1.8 Host immune response to HCMV

1.8.1 Innate immune response

HCMV establishes a life-long association with its host. The virus remains in a latent form within a healthy individual, but can cause severe disease when the immune system is impaired. This highlights the immune system's crucial role in controlling viral replication. The immune response to any invading organism consists of two branches, the innate response and the adaptive response. The first occurs immediately after infection and is mediated mainly by macrophages (Janeway *et al*, 1996). Macrophages function at the front line of immune defences against incoming pathogens. The invading organism is phagocytosed by macrophages, broken down and presented to the cells constituting the adaptive immune response. Macrophages

can inhibit virus replication in adjacent cells themselves, by secreting α, β and γ interferons (Gessani *et al*, 1998). They also secrete TNF-a and interleukins, which attract neutrophils and natural killer (NK) cells (Wewers *et al*, 1997), leading to elimination of foreign pathogen. Natural killer cells function as both effector cells and as a source of cytokines. They can lyse transformed cells and virus-infected cells, becoming functional in the absence of MHC class I antigens on target cells. Their importance is emphasized in patients devoid of NK cells but with an otherwise normal T cell and antibody response, who exhibit increased susceptibility to HCMV (Biron *et al*, 1989). A number of other correlations between NK cell function and sensitivity to CMV infection have been reported *in vivo* (Cauda *et al*, 1987). For example the ability of bone marrow transplant patients to develop an NK cell response correlated with recovery from HCMV infection (Quinnan *et al*, 1982). Studies have shown that mice genetically deficient in functional NK cells have an increased susceptibility to murine CMV (Shellam *et al*, 1981). Additionally, in mice with severe combined immunodeficiency, NK cells could decrease levels of HCMV replication, without being able to completely inhibit infection, suggesting that T and B cell responses are also required for the eradication of infection (Welsh *et al*, 1994). Therefore, NK cells represent a bridge between innate and adaptive immune response, by inhibiting the spread of infection until specific immune responses are developed (Biron *et al*, 1989, Kos *et al*, 1996).

There are two types of interferons (IFNs), IFN type I (IFN-α and IFN-β) and IFN type II (IFN-γ). Mammalian cells respond to infection by generating an intracellular antiviral state, characterised in many cases by the synthesis and secretion of type I interferons. There are at least 20 types of IFN-α, produced mainly by infected monocytes and macrophages. Fibroblasts also produce some type I IFNs. Most of the viruses studied, including HCMV, show evidence of inducing an IFN-based antiviral response through the induction of IFN-α, IRFs and STAT-1 (Zhu *et al*, 1997). In the case of HCMV, this induction is principally mediated by the interaction of gB with a receptor on the surface of the target cell (Boyle *et al*, 1999). Type I IFNs are produced within 24 hours after infection, and bind to receptors on neighbouring cells. They then activate intracellular signalling pathways that lead to the binding of transcription complexes to IFN-responsive elememts (ISRE) for IFNs, activating transcription of

IFN stimulated genes (ISGs) and inducing an antiviral state. In addition, they up-regulate MHC class I expression on neighbouring uninfected cells and activate NK cells, causing enhanced killing of virus-infected cells.

1.8.2 Adaptive immune response

The innate response is not successful and some infectious agents, including HCMV, can persist or replicate in macrophages. It is at this point where the cellular and humoral immune responses become essential in host control of HCMV infection.

Cellular immune response

The cellular immune response is essential for restraining CMV infection. This is demonstrated by the fact that acute disease is seen in people who have severely impaired cell-mediated immunity, such as patients who have AIDS or undergo bone marrow transplantation. $CD8^+$ cytotoxic T-lymphocytes (CTLs) are the major cell-mediated immune response to HCMV. They recognize a specific antigen only when presented by MHC class I antigen on the target cell and eliminate it. The induction of a CTL response is dependent on recognition of specific T-cell epitopes by the immune system. Several different HCMV antigens have been identified to generate a cytotoxic T cell response but the protein responsible for the majority of the response is pp65 (ppUL183) (Boppana and Britt, 1996, Wills *et al*, 1996). Many pp65-derived peptides are targets for CTLs, however one HLA-A2 restricted peptide seems to be immunodominant (Solache *et al*, 1999). pp150, another tegument protein, is also recognised by CTLs (Li *et al*, 1997). The fact that the majority of the CTL response is directed against structural virion proteins ensures that the virus is presented to the immune system prior to the initiation of its replication, allowing it to be eliminated before producing virus progeny. Unfortunately in cells that reactivate latent virus, pp65 and pp150 cannot be processed until after the onset of viral replication, rendering these cells relatively resistant to elimination by pp65- and pp150-specific CTLs (Khattab *et al*, 1997). Some IE proteins also act as T cell epitopes. Recently, the presence and functional capacity of CMV-specific $CD8^+$ T cells was monitored in organ transplant patients and healthy donors with latent CMV infection. The study showed that although the CMV-specific T cell numbers were similar in both groups,

the $CD8^+$ T cells in immunosuppressed patients exhibited a reduced functional response (Engstrand *et al*, 2003). These findings indicate impaired activation of cytotoxic T cells controlling latent infection in immunosuppressed individuals.

The murine CMV-infected mouse model has provided a large amount of information on immune responses to infection. Early studies showed that the suppression of T cell function led to reactivation and dissemination of natural infection (Gardner *et al*, 1974). Another study using mice immunosuppressed with irradiation, showed an increased virus growth rate and mortality, compared to non-irradiated mine (Reddehase *et al*, 1985). Recently it has also been shown that the adoptive transfer of $CD8^+$ T cells in murine bone marrow recipients can prevent lethal CMV disease (Steffens *et al*, 1998). Finally, the adoptive transfer of murine CMV-immune lymph node cells in T-cell depleted mice can prevent the development of retinitis (Lu *et al*, 1997).

Adoptive transfer of HCMV-specific T cell clones has been used as a therapeutic strategy in bone marrow transplant patients, after observing an association between CMV-specific CTL recovery and recovery from HCMV pneumonia. The transfer of CTL clones prevented the development of HCMV viraemia and disease and the persistence of $CD8^+$ T cells required HCMV-specific $CD4^+$ T helper cell function (Walter *et al*, 1995). $CD4^+$ T lymphocytes recognize a specific antigen only when presented by MHC class II antigen on the antigen-presenting cell. Unlike MHC class I antigens, which are present on all cell types, MHC class II antigens only exist on B cells, dendritic cells and macrophages. In humans with HCMV infection the presence of $CD4^+$ helper T lymphocyte responses and their protective role has been demonstrated. In response to CMV infection these cells are able to release cytokines to promote viral clearance. Lymphocytes from healthy seropositive volunteers could mediate the lysis of HCMV-infected monocytes, suggesting a direct antiviral effect (Lindsley *et al*, 1986). Additionally, they have been shown to be essential for the $CD8^+$ cytotoxic response (Hayward *et al*, 1984).

Humoral immune response

The major impact of the humoral immune response is the eventual clearance of virus from the body. Effective antibody responses comprise the induction of neutralising antibodies and antibodies that can mediate antibody-dependent cytotoxicity. There is evidence that humoral immunity to CMV is crucial for the prevention of disease in the host. Data have shown that in immunosuppressed mice infected with murine CMV, dissemination was reduced significantly by administering CMV-specific antibodies (Shanley *et al*, 1981). Furthermore, in the guinea pig model, immunisation of pregnant dams with CMV-specific antibodies resulted in a decrease in the rate of foetal infection after CMV challenge (Harrison *et al*, 1995). In humans, the protective role of humoral immunity has been clearly demonstrated during pregnancy, where the presence of maternal antibody to HCMV before conception was shown to provide significant protection against HCMV disease in the infant (Fowler *et al*, 1992). Numerous studies have provided evidence that serum antibodies to HCMV obtained before transplantation are protective against severe HCMV disease (Alberola *et al*, 2000). Renal transplant patients receiving pretransplant immunisation exhibited a reduced severity of HCMV disease (Plotkin *et al*, 1984). Additionally, passive immunisation of seronegative renal transplant patients using hyperimmune γ-globulin has also provided a degree of protection against HCMV disease in controlled trials, with a reduced incidence of CMV associated fever, and mortality (Snydman *et al*, 1987, Weimar *et al*, 1990). Antibodies to a wide variety of immunogenic HCMV proteins can be detected in sera from seropositive humans (Landini *et al*, 1988). Nearly all the immune human sera have antibodies to envelope glycoproteins gB and gH, and to the tegument phosphoprotein pp150 (Schoppel *et al*, 1998). However, neutralising antibody is directed mainly against gB and gH (Kniess *et al*, 1991).

1.8.3 Immune escape mechanisms

The success of HCMV as a long-term parasite is in part due to its ability to interfere with the immune response. The virus has evolved multiple strategies for immune escape at different times of its replication cycle. There are two classes of viral proteins mediating immune regulation. The first class comprises proteins encoded by

genes with sequence homology to cellular genes. This suggests that the virus has "stolen" genes from the host that were subsequently modified through mutation or selection to benefit the virus. The second class contains proteins encoded by viral genes without sequence similarity to host genes. These may represent an example of co-evolution and might possess properties required for interaction with the cellular machineries. An overview of mechanisms that HCMV uses to evade the immune response follows.

Modulation of MHC molecules

One HCMV antiviral strategy involves the modulation of MHC class I molecules, resulting in inhibition of antigen presentation and so clearance by $CD8^+$ CTLs. HCMV down-regulates MHC class I on the surface of infected cells (Barnes and Grundy, 1992), which has been shown to correlate with increased resistance to lysis by CTLs (Beersma *et al*, 1993, Warren *et al*, 1994). A series of elegant studies *in vitro* has shown that the viral genes mediating this effect are US2, US3, US6 and US11 (Jones *et al*, 1995). US3, an IE gene, prevents MHC class I cell surface expression by retaining the $\beta_2$microglobulin-associated heavy chain in the endoplasmic reticulum (ER) immediately after infection (Jones *et al*, 1996). US2 causes decreased levels of the class I heavy chain, by binding to it and transporting it to the proteasome for degradation (Jones *et al*, 1997). US6 inhibits peptide transport to the ER lumen by binding to TAP1/2 system, which are essential for antigen translocation (Ahn *et al*, 1997). US11 acts in a similar manner to US2 (Wiertz *et al*, 1996), but probably targeting a different class I allele. The region responsible for MHC class I degradation is located at the transmembrane part of the protein (Tomazin *et al*, 1999). HCMV also inhibits class I presentation at a different stage, via the tegument protein pp65. pp65 phosphorylates the CMV IE proteins, disrupting their processing by the proteasome and their presentation to CTLs (Gilbert *et al*, 1996). Recently, an additional viral gene with a role in MHC class I regulation was identified. The HCMV UL82 gene product, termed pp71, is a tegument phosphoprotein that localises at nuclear domain 10 (ND10) structures and is required for viral gene transcription. Pp71 has been shown to increase infectivity of viral DNA and degrades hypophosphorylated forms of retinoblastoma (Rb) proteins. Cell surface MHC class I levels were significantly

decreased by 48 hours after infection with adenovirus expressing pp71, whereas total cellular levels were similar in infected and uninfected cells (Miller *et al*, 2002).

HCMV can also modulate cell surface expression of MHC class II antigens. Infection of macrophages with HCMV resulted in a down-regulation of cell surface MHC class II expression. Furthermore, the virus was shown to prevent induction of MHC class II on infected endothelial cells (Knight *et al*, 1997). More recently, it was reported that HCMV infection of glioblastoma cells stably transfected with the major class II transcriptional regulator (CIITA), resulted in a decrease in cells surface MHC class II levels (Cebulla *et al*, 2002). This effect was not due to a decrease in biosynthesis of MHC class II complex components, or due to their degradation by a proteasome-dependent mechanism (Colleen *et al*, unpublished data). The loss of MHC class II expression correlated with a restricted perinuclear localisation of MHC class II molecules and a disruption of actin and microtubule networks. Furthermore, transfection of cells with cosmids spanning the genome suggested that the UL131 to US25 region also led to decreased accumulation of MHC class II at the cell surface. These data imply that the HCMV could alter the localisation and transport of MHC-II complexes to perinuclear endosomal/lysosomal compartments, leading to down-regulation of $CD4^+$ T cell responses.

There are a number of contradictory *in vitro* studies, showing an increased MHC class I expression after CMV infection of fibroblasts (Grundy *et al*, 1988), endothelial (Tuder *et al*, 1994), epithelial (Van Dorp *et al*, 1993) and smooth muscle cells (Hosenpud *et al*, 1991). This up-regulation of MHC class I molecules on the surface of infected cells requires the binding of HCMV particles to cell surface heparan sulphate proteoglycans (Song *et al*, 2001). *In vivo* studies also suggest that CMV infection increases the expression of class II molecules on infected cells. This was seen in tubular and endothelial cells of human renal allograft recipients with an active CMV infection (Von Willebrand *et al*, 1986). The same effect was observed on endothelial cells in the kidney and liver (Ustinov *et al*, 1994) in adult rats. Interestingly, the increased expression of MHC molecules on infected cells could elicit $CD4^+$ and $CD4^+$ cell activation, contributing to allograft rejection. Indeed, increased class I expression was observed on hepatocytes, epithelial and endothelial cells during liver allograft failure (Gouw *et al*, 1988) and increased class II expression

is associate with acute rejection in kidney transplant recipients (Von Willebrand *et al*, 1993).

Induction of FcγR

HCMV induces the expression of Fcγ receptors on infected cells (Litwin *et al*, 1990). These receptors can bind nonvirus-specific antibodies, inhibiting the attachment of specific IgG to the cell, and so protecting infected cells from antibody-mediated destruction (Adler *et al*, 1978). HCMV induces such a receptor soon after infection, which could allow the virus to evade the immune response (Keller *et al*, 1976, Sakuma *et al*, 1977). Additionally, HCMV induces an Fcγ receptor in fibroblasts and endothelial cells that is distinct from the human cellular Fcγ receptors (MacCormac and Grundy, 1996). The increased expression of the FcγR could also lead to deposition of immune complexes on the infected cell, a process which could contribute to the initial lesions seen in the development of atherosclerotic plaques (Raines and Ross, 1993) or tissue injury (Oldstone *et al*, 1975).

Escape from NK cell lysis

Although the downregulation of MHC class I expression prevents $CD8^+$ CTL recognition, virally infected cells that downregulate these molecules are susceptible to lysis by NK cells (Leong *et al*, 1998). NK cells have receptors for certain MHC molecules. Some of these receptors silence the NK cytolytic machinery and act as killer cell inhibitory receptors (KIR). Other receptors, named leukocyte immunoglobulin-like receptors (LIR), are expressed mainly on monocytes and B cells. Studies have shown that HCMV may protect cells from NK cell elimination. Evidence supporting this came from the observation that HCMV-infected endothelial cells with reduced MHC class I expression were resistant to NK lysis (Waldman *et al*, 1998). In 1988, it was demonstrated that HCMV encodes a MHC class I homologue, named UL18 (Beck and Barrell, 1988). There is contradictory data about the function of UL18. It was initially thought to block NK cell activity through binding with the killer inhibitory receptor on NK cells (Reyburn *et al*, 1997), but was later discovered that UL18 did not appear to bind this inhibitory receptor (Leong *et al*, 1998). The role

of UL18 in NK evasion is still unclear. Recently, however, it was demonstrated that UL18 was required for the induction of T cell unresponsiveness and for the growth inhibition of leukaemic T cell lines. These data suggest that this protein may participate in the silencing of T cells, leading to subversion of the immune system (Odeberg *et al*, unpublished data).

In 2000, Tomasec *et al* identified an MHC-I-like region of nine amino acids in another viral protein, UL40 (Tomasec *et al*, 2000), which was conserved in multiple isolates of CMV. This protein was able to up-regulate HLA-E cell surface expression by binding to inhibitory receptors on the surface of NK cells, providing a mechanism for immune evasion. This effect was solely mediated by the MHC-I-like, nine amino acid region of UL40. The role of UL40 in NK cell evasion was confirmed in 2002, where AD169 was shown to abolish the cytotoxicity of NK cells, while this effect was inhibited when using an AD169, UL40 deletion mutant (Wang *et al*, 2002).

However, clinical isolates of HCMV confer a much stronger NK resistance than the laboratory strains tested so far, and the resistance was not related to MHC-I expression (Cerboni *et al*, 2000). This raised the question whether any proteins from the UL/b' region of clinical strains could function as ligands for NK receptors. A program for prediction of protein function identified UL142 as a UL18 / MHC class I homologue. mRNA expression analysis in infected fibroblasts classified UL142 as a late gene (Wills *et al*, unpublished data). By 72 hours, fibroblasts infected with Toledo were resistant to NK lysis, while fibroblasts infected with AD169 were lysed even when the level of MHC class I down-regulation was comparable. Further experiments showed that fibroblasts infected with a UL142 knockout virus were more susceptible to polyclonal NK lysis. Therefore, UL142 represents another viral protein that can inhibit the lytic activity of some NK clones. UL141 is another UL/b' region gene, expressed by 24 hours after infection of fibroblasts with HCMV and retained within the cell. Studies have shown that UL141 is a robust inhibitor of NK cell function by an unknown mechanism. These data suggest that the loss of the UL/b' region after serial propagation of clinical isolates in culture, could be associated with the loss of NK-specific functions.

HCMV UL16 is another gene that interferes with NK cell function. It was described in 1992 as a late gene, dispensable for viral growth and replication. Its gene product is soluble and has been shown to bind some of the ligands recognised by an activating receptor on NK cells, named NKG2D (Bauer *et al*, 1999). These ligands include UL-16 binding proteins (ULBP) 1 and 2 and MICB (Cosman *et al*, 2001). Cell surface expression of these ligands after HCMV infection is reduced (Rolle *et al*, unpublished data) and correlated with an increased dependency on NKG2D for recognition, with the overall NK sensitivity remaining the same. Infection with a UL16 deletion mutant however, resulted in an entirely NKG2D-dependent increase in NK cell sensitivity. Consequently, a new model of immune evasion for HCMV has been proposed. Following viral infection, the host cell initiates ULBP expression in order to increase its susceptibility to NKG2D-mediated NK recognition. This antiviral response is however limited by UL16, which interferes with ULBP1 and 2 expression, leading to escape from NK cells.

In murine CMV, it has been shown that the product of M157 gene serves as a ligand for an activating NK cell receptor, Ly49H (Arase *et al*, 2002, Smith *et al*, 2002). The significance of this protein for NK cell-mediated virus control was also tested *in vivo*, using deletion mutants on both Ly49H positive and negative mouse strains (Bubic *et al*, unpublished data).

CMV –encoded homologues of IL-10, TNF R, chemokines and chemokine receptors

Cytokines play a key role in the initiation and regulation of the innate and adaptive immune response, and viruses have learned evolved strategies to modulate cytokine production, and signal transduction (Miskin *et al*, 1998). Interleukin 10 is a cellular cytokine that down-regulates MHC class I and II expression and inflammatory cytokine production (Fumeaux and Pugin, 2002, Haddad *et al*, 2003). HCMV encodes a viral protein expressed in fibroblasts, which shows 27% sequence homology to IL-10, termed vIL-10 (UL111a) (Kotenko *et al*, 2000). Despite this low level homology, cmvIL-10 was found to compete for binding to the cellular IL-10 and so could contribute to the immunosuppression observed during HCMV infection. The viral IL-10 also suppressed the maturation and cytokine production of DCs, preventing the polarisation of naïve CD4+ T cells to IFN-producing T helper cells. Further studies

have shown that the presence of vIL-10 during stimulation of immature DCs with LPS strongly inhibited their maturation and secretion of pro-inflammatory cytokines such as IL-12 (Chang *et al*, unpublished data). These data are consistent with a role for vIL-10 in reducing tissue inflammation and DC migration to lymph nodes. Furthermore, mature DCs treated with vIL-10 failed to induce MHC class II expression compared to untreated mature DCs. These data suggest that HCMV can attenuate the ability of DCs to induce immune responses, leading to the establishment of persistent infection. Additionally, infection of fully permissive astrocytes with HCMV resulted in vIL-10 mRNA expression (Cheeran *et al*, 2003). Medium from the infected astrocytes inhibited virus-induced IL-10 production from microglial cells through the IL-10 receptor, suggesting another mechanism employed by HCMV to avoid elimination by the immune system.

Chemokines are small cytokines that mediate recruitment of leukocytes to sites of infection and inflammation (Baggiolini *et al*, 1997, Greaves and Schall, 2000). A number of DNA viruses have been shown to encode α (CXC) and β (CC) chemokines and chemokine receptors, resulting in immune modulation (McFadden *et al*, 1998). HCMV encodes US28, a protein with homology to a β (CC) chemokine receptor (Gao and Murphy, 1994), which enters infected cells through clathrin-mediated endocytosis (Fraile-Ramos *et al*, 2003). US28 mRNA is detected before gB and pp65 transcripts and is present in permissive, semi-permissive and non-permissive cell types (Zipeto *et al*, 1999). The protein was shown to bind RANTES and the monocyte chemoattractant protein 1 (Kuhn *et al*, 1995, Vieira *et al*, 1998). This binding could lead to increased calcium levels in the cells and provide a favourable environment for viral gene expression (Neote *et al*, 1993). In 1999, it was shown that US28 was required for the migration of HCMV-infected smooth muscle cells. More recent studies indicated that US28 promotes cellular migration through the activation of a focal adhesion kinase (FAK) signalling complex and promotes a mechanism by which HCMV contributes to the development of vascular disease (Streblow *et al*, 1999).

HCMV encodes two α chemokine homologues, UL146 and UL147 (Penfold *et al*, 1999), which are conserved in clinical isolates. UL146 was shown to induce calcium mobilisation and neutrophil chemotaxis and degranulation. Although the function of

this protein has not been elucidated, it could contribute to the disruption of normal leukocyte migration and to viral dissemination by infecting neutrophils. Additionally, HCMV UL144 has been shown to be homologous to members of the tumour necrosis factor receptor (TNFR) superfamily (Benedict *et al*, 1999, Lurain *et al*, 1999). UL144 was expressed early after infection and was retained in an intracellular compartment. This gene product is important because of its possible role in HCMV pathogenesis as a growth factor decoy that facilitates evasion of the immune response by the virus. Sequence analysis of guinea pig CMV predicted an ORF of 101 amino acids with homology to the CC chemokines (Haggerty and Schleiss, 2002). This ORF had the strongest sequence identity to the macrophage inflammatory protein (MIP) subfamily, and was named GPCMV-MIP. Using recombinant GPCMV-MIP, specific signalling was observed with the CCR1 receptor and further cell migration assays indicated that this protein could induce chemotaxis in CCR1 cells. Finally, a GPCMV-MIP deletion mutant replicated normally in cell culture, but was attenuated compared to wild type virus after inoculation in guinea pigs at early time points post-infection.

Interferon modulation

The key role of IFN type I and II as one of the first lines in the anti-viral defence system is highlighted by the fact that viruses employ a variety of anti-IFN strategies (Goodbourn *et al*, 2000). Viruses can block IFN-induced transcriptional responses and the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signal transduction pathways. Additionally, viruses inhibit IFN effector pathways that induce an antiviral state in the cell and reduce virus replication. HCMV has been shown to inhibit the JAK/STAT pathway by reducing levels of JAK1 and p48 (Goodbourn *et al*, 2000). Experiments with mouse CMV indicate that the gene M27 interferes with IFN signalling. Isolated expression of M27 prevented the nuclear accumulation of STAT2 in infected cells and resulted in a rapid proteasome-dependent degradation of STAT2 only. M27 was also found to have a more drastic effect on IFN gamma mediated antiviral activities, which was dependent on STAT2 along with IFN alpha/betaR1 expression (Zimmermann *et al*, unpublished data). These data support a model of crosstalk between IFN gamma and IFN alpha/beta signalling components, which requires the presence of STAT2.

Modulation of cell adhesion molecules

Alterations to cell adhesion molecules have been associated with leukocyte extravasation during graft rejection and atherosclerosis (Hayry *et al*, 1992). Several studies have suggested that increased expression of cell adhesion molecules may result in prolonged inflammatory reactions in the allograft after HCMV infection (Eriksson *et al*, 2001). Indeed, in heart transplant patients there is an increase of ICAM-1 and VCAM-1 levels on endothelial cells, associated with T cell infiltration during allograft rejection (Briscoe *et al*, 1991). Another adhesion molecule, E-selectin, is also up-regulated in heart transplant patients with CMV viremia (Koskinen, 1993). Additionally, the levels of vascular adhesion protein-1 (VAP-1) were increased in acute rejection liver allografts in RCMV-infected rats (Martelius *et al*, 2000). Using the same model, a prolonged expression of ICAM-1 and VCAM-1 in the CMV infected grafts was associated with accelerated chronic allograft nephropathy (Kloover *et al*, 2000). Up-regulation of E-selectin, ICAM-1 and VCAM-1 was also observed on HCMV-infected endothelial cells *in vitro* (Span *et al*, 1991, Shahgasempour *et al*, 1997, Altannavch *et al*, 2002), while increased levels of ICAM-1 and LFA-3 have been demonstrated on CMV-infected fibroblasts *in vitro* (Grundy and Downes, 1993). Other *in vitro* data using endothelial cells however, have failed to confirm the alterations in levels of cell adhesion molecules (Sedmak *et al*, 1994). Further studies are required in order to provide a clear picture of the functional consequences of the expression of adhesion molecules on the surface of endothelial cells after HCMV infection.

Inhibition of apoptosis and complement-mediated lysis

Apoptosis, or programmed cell death, can be triggered by TNF ligands, cell cycle inhibitors and viruses. Among other functions apoptosis acts as an innate cellular response to limit viral propagation and viruses encode proteins that can inhibit apoptosis (Smith *et al*, 1997, Tortorella *et al*, 2000). HCMV also uses this strategy to prolong the lifespan of infected cells and therefore maximises the chance to spread the infection. It has been shown that infection of fibroblasts with HCMV inhibited death receptor-induced apoptosis. CMV UL37, localised in mitochondria, was identified as the viral gene mediating this effect (Goldmacher *et al*, 1999). Additionally, the IE1

and IE2 HCMV genes have been shown to interfere with TNF-induced apoptosis (Hengel *et al*, 1998, Kalvakolanu, 1999). Another viral anti-apoptotic mechanism is targeting the cellular proteins implicated in the control of apoptosis. Some examples include inhibition of cascade activation, encoding homlogues of cellular anti-apoptotic protein Bcl-2 and inactivation of p53 (Macen *et al*, 1998, Wang *et al*, 2001). HCMV also contains two genes, UL36, UL37, which encode proteins with anti-apoptotic functions, named vICA and vMIA, respectively (Skaletskaya *et al* 2001, McCormick *et al* 2003).

The complement system is also major innate host defence mechanism (Kotwal, 2000). Viruses encode homologues of complement regulatory proteins that block complement activation and neutralisation of virus particles. HCMV employs a clever tactic in order to avoid complement attack. HCMV actively acquires cellular CD59, a complement inhibitor, and incorporates it into the viral envelope (Hengel *et al*, 1998). CD46 is another complement inhibitor, expressed in cells, protecting them from homologous complement lysis. Infection of these cells with murine CMV resulted in increased expression of CD46 mRNA and protein levels (Nomura *et al*, 2002). This represents another strategy by which murine CMV can circumvent host complement attack. Table 1.3 summarises the strategies by which HCMV evades immune recognition.

Function	Gene / Protein	Proposed mechanism
Inhibition of MHC class I antigen presentation	ppUL183	Blocks IE-1 peptide presentation
	US2, US11	Dislocate class I to cytosol
	US3	Retains class I in the ER
	US6	Inhibits TAP-dependent peptide translocation
	UL82	Unknown
Inhibition of apoptosis	UL122, UL123	Inhibit TNF-induced apoptosis
	UL37	
Inhibition of membrane attack complex (MAC) formation	Host CD59	Host protein incorporated into envelope
	Host CD46	Increased protein levels on the surface of infected cells
Inhibition of JAK / STAT pathway	Unknown	Decrease in the levels of JAK1 and p48; proteasome involvement
TNF receptor	UL144	TNF R homologue
β chemokine receptor	US28	Mediates migration of smooth muscle cells and decreases local concentration of RANTES
α chemokines	UL146, UL147	Chemoattractants for neutrophils
IL-10	UL111	IL-10 activity Immunosuppressive
Effect on NK cells	UL18,UL40, UL142	MHC class I homologues Inhibit NK-mediated cell lysis
	UL16	Interferes with cell surface expression of ligands that bind an NK activation receptor
	UL141	unknown
Inhibition of apoptosis	UL36	Inhibits caspase 8 activation
	UL37	Disrupts mitochondrial fission

Table 1.3. The inhibition of immune responses by HCMV.

1.9 Pathology and treatment of HCMV disease

1.9.1 Factors in disease production

The pathogenesis of HCMV infection is complex and is determined by a variety of factors, mainly the type of infection, the level of viremia, the virus load and patient group.

Type of infection

The type of HCMV infection, namely primary infection, reinfection or reactivation, are important in determining the severity of HCMV disease. Infection in an immunologically naïve individual represents the greatest risk of disease in pregnant women. Primary infection of the mother has a foetal transmission rate of 50% compared to less than 2% for recurrent infection. Additionally, it is quite rare for infants to develop clinically apparent HCMV disease when the mothers have pre-existing immunity to the virus (Fowler *et al*, 1992). In bone marrow transplant patients, reactivation is the major source of HCMV disease (Winston *et al*, 1985) while in solid organ transplant patients, reinfection of seropositive individuals represents more of a risk for disease than reactivation of latent virus (Grundy *et al*, 1988).

Viremia

The detection of HCMV in the blood (viremia) as a marker of disease is more predictive that the detection of HCMV in saliva or urine (Gerna *et al*, 1991, Kidd *et al*, 1993). Additionally, a recent study looking at allogeneic peripheral blood stem cell transplant patients showed that reconstitution of total lymphocytes was associated with recovery from viremia and no progression to HCMV disease (Gutierrez *et al*, 2003). Viremia is an indicator of overwhelming virus replication at local sites of infection, resulting from an imbalance between the rate of HCMV production at sites of infection and the immune clearance rate, which permits HCMV to spread to multiple organs (Rubin, 1991). The detection of plasma HCMV DNA (cell-free viremia) in seroconverting blood donors may explain the low residual risk of CMV

transmission by both CMV seronegative and leukodepleted seropositive blood (Drew *et al*, 2003).

Virus load

The advent of PCR has enabled greater sensitivity of HCMV detection and quantitation of HCMV load. The association between the quantity of virus load in the urine of congenitally infected infants and disease was first established in 1975 (Stagno *et al*, 1975). The results from this study showed a significant association between disease and virus load in the urine at birth of congenitally infected infants compared to asymptomatic infected infants. Additional studies have also found a direct relationship between elevated virus load and disease in a variety of immunocompromised patient groups (Fox *et al*, 1995, Bowen *et al*, 1996, Cope *et al*, 1997, Emery *et al*, 1999, Hassan-Walker *et al*, 1999, Smith *et al*, 1999, Spector *et al*, 1999, Emery *et al*, 2000, Nokta *et al*, 2002, Boeckh *et al*, 2003).

1.9.2 Transplant patients

Solid organ transplantation

In the absence of prophylaxis, the onset of HCMV infection in transplant recipients of organs such as kidney, liver, lung, and heart, usually occurs within the first three months after transplantation, as indicated by detection of virus in blood or virus shedding (Falagas and Snydman, 1995, Ducloux *et al*, 1997). The onset and establishment of disease can vary, depending on the degree of immunosuppression, initiation of antiviral treatment, allograft source, HLA mismatching, receipt of infected blood products and donor seropositivity. Additionally, symptomatic disease usually results from primary infection rather than reactivation (Ho *et al*, 1975, Betts *et al*, 1977). There is a lower risk of HCMV disease resulting from virus reinfection, which is associated with the transplantation of an organ from a seropositive donor to a seropositive recipient, suggesting that pre-existing immunity in the recipient is beneficial, leading to a reduction in the incidence of HCMV disease. This observation is consistent with the reduced growth rate, basic reproductive number and peak virus load in solid organ transplant patients who are D+R+ (Emery *et al*, 2002). HCMV seropositive blood products, which may be given as part of the transplantation

program, are also an important source of virus transmission for HCMV seronegative transplant recipients. Multiple transfusions have been associated with HCMV infection (Chou *et al*, 1987, Landaw *et al*, 1996) and procedures that limit the quantity of leukocytes in transfused blood limit the incidence of HCMV infection following transfusion, suggesting that leukocytes are responsible for transfusion-associated HCMV infections in transplant recipients (Gunter, 1995). Patients that received high doses of cytotoxic drugs showed increased morbidity from HCMV infection (Hayry *et al*, 1992). In the case of renal transplant recipients, the degree of HLA mismatch increases the risk for HCMV infection (Boland *et al*, 1993, Keever-Taylor *et al*, 2001).

The most common manifestations of HCMV disease are fever, leukopenia, thrombocytopenia, malaise and macular rash. Some patients however may develop life-threatening diseases like pneumonitis, GI (gastrointestinal) tract ulceration, hepatic dysfunction, and fungal and bacterial infections (Duncan *et al*, 1994). Host versus graft rejection together with an active HCMV infection is also associated with severe disease and poor prognosis (Goral and Helderman, 1994, Martelius *et al*, 1997). HCMV infection has been consistently associated with poor graft survival, although the mechanism underlying the rejection process is not well understood. A potential mechanism is the upregulation of MHC molecules in the organ, leading to increased immunogenicity. Retinitis in transplant recipients is very rare.

Bone marrow transplantation

The greatest risk of HCMV disease in seropositive bone marrow transplant recipients is associated with reactivation of endogenous HCMV rather than re-infection of exogenous virus (Winston *et al*, 1985). Additionally, patients receiving bone marrow from seropositive donors show a lower incidence of HCMV disease (Grob *et al*, 1987, Lin *et al*, 2002). These findings indicate that instead of marrow representing a high risk for virus transmission, it may actually provide immunity to the recipient. Furthermore, the use of screened, seronegative blood products has also decreased the occurrence of primary infection or re-infection in these patients (Bowden *et al*, 1991).

Pneumonitis is the major life-threatening presentation of HCMV in bone marrow (BM) transplantation, with an 85% mortality rate if left untreated (Meyers *et al*, 1986). For allogeneic transplants, about 0% to 15% of seronegative recipients of marrow from a positive donor and 6% to 36% of seropositive patients develop pneumonitis (Boeckh and Bowden, 1995). This complication is much less common in autologous transplant patients, consistent with the supposed immunopathologic nature of the disease process (Grundy *et al*, 1987). While HCMV excretion is common after autologous or identical twin marrow transplant, HCMV disease is not. Furthermore, graft-versus-host disease (GVHD) occurs more frequently in the case of donor-recipient histoincompatibility. In 1996, a multivariate analysis of risk factors for HCMV disease was performed, showing that GVDH was the most significant risk factor for pneumonitis and mortality (Ljungman, 1996). The induction of GVDH by HCMV could either result from uncontrolled virus replication in the lung or from an immunopathological reaction triggered by HCMV infection (Greenberg, 1991). Despite the proven associations between HCMV infection and GVDH, the actual mechanism by which HCMV mediates GVDH remains unclear. Studies using the murine CMV model support the hypothesis that the pneumonitis resulting from GVHD is immune-mediated (Grundy *et al*, 1987). Experiments looking at pneumonitis after bone marrow transplantation in mice showed that while there was no histopathological evidence of the disease, the virus could still replicate in the lungs of these mice (Shanley, 1991) and disease resulted after altering the immune response. In another model, pneumonitis only developed in mice infected with murine CMV and given a graft-versus-host challenge (Grundy *et al*, 1985). Therefore in the mice, GVHD represents a risk factor for pneumonitis. Additionally, the infiltration of T cells in mice contributes to the pathology of CMV disease. Other, less common, diseases in bone marrow transplant recipients include GI tract disease, hepatitis, encephalitis and retinitis. Retinitis however has become more common since more matched unrelated donor transplants have occurred, probably due to a delayed immune reconstitution compared to sibling transplants (Larsson *et al*, 2002).

In the case of BM transplant patients, the amount of HCMV in blood (whole blood or peripheral blood leukocytes) and bronchoalveolar lavage fluid is a strong predictor of disease (Weinberg *et al*, 2002). Successful management of HCMV infection in these patients requires pre-transplant donor and recipient screening, prevention of HCMV

transmission through blood products, virological and clinical screening for HCMV infection after transplantation, and the correct use of antiviral therapy.

1.9.3 HIV patients

HCMV infection is an important pathogen in HIV positive individuals (Drew, 2003). Clinical disease due to HCMV has been reported in up to 40% of patients with advanced human immunodeficiency virus (HIV) disease (Cheung and Teich, 1999). The risk of HCMV increases with decreased immune function, and symptomatic HCMV disease is most frequently associated with a low $CD4^+$ T cell count (<50 cells per mm^3) (Gallant *et al*, 1992, Pertel *et al*, 1992). The majority of homosexual men are seropositive for HCMV (Collier *et al*, 1987), suggesting that disease in these patients is most likely due to reactivation of endogenous virus. Re-infection with new HCMV strains though may also play a role in HCMV pathogenesis, since many AIDS patients are infected by more than one HCMV strain (Drew and Mintz, 1984, Collier *et al*, 1989).

HCMV disease has been classified as an AIDS defining illness according to the Centre for Disease control in Atlanta, USA. It has also been suggested as a cofactor in the development of AIDS, because clinical and molecular studies have linked CMV and the progression of HIV-1 infection (Webster *et al*, 1989, 1992, Mocroft *et al*, 1999). Epidemiological studies have shown that the progression to AIDS occurs much faster in patients infected with both viruses (Detels *et al*, 1994). *In vitro* evidence also indicate that HCMV can enhance HIV-1 replication or reactivation from infected cells. Syncytiotrophoblasts, for example, are restrictive for HIV-1 replication, however when these cells are co-infected with HIV-1 and HCMV, there is a 1500-fold increase in HIV-1 replication (Toth *et al*, 1995). In monocyte-derived macrophages, HCMV also enhances HIV replication (Lathey *et al*, 1994). HCMV can enhance HIV-1 replication through a number of mechanisms, including the induction of a Fcγ receptor expression (McKeating *et al*, 1990), transactivation of the promoter in the HIV-1 long terminal repeat region (Barry *et al*, 1990) and production of proinflammatory cytokines (Ostrowski *et al*, 1998).

Unlike the clinical manifestations in the transplant patients, the most common symptom in AIDS patients is chorioretinitis, which is seen in 20% to 45% of individuals (Jacobson *et al*, 1988). This condition often leads to blindness due to necrotising damage to the retina. HCMV can also cause encephalitis, characterised either by damaging of the cortex of the brain or by damaging the cranial nerves (Kalayjian *et al*, 1993), leading to delirium and rapid progression to death. Apart from the central nervous system (CNS), HCMV disease can also affect the peripheral nervous system, causing polyradiculopathy as a result of virus infection of the spinal cord (Whitley *et al*, 1998). GI tract disorders, characterised by ulcerations or epithelial lining perforations, are the second most common clinical syndrome, occurring in 5% to 12% of HIV infected individuals (Williams *et al*, 1992). Symptoms include oesophagitis, gastritis and enterocolitis. Endothelial, epithelial and smooth muscle cells are the major cell types infected (Sinzger *et al*, 1995). The GI tract damage is thought to result from direct viral cytopathogenicity, since the symptoms are resolved after anti-viral treatment (Dieterich *et al*, 1988). Other syndromes caused by HCMV include fever, hepatitis and polyradiculopathy (Bell, 1998). HCMV pneumonitis is less common in AIDS patients than in transplant recipients, suggesting that the pathogenesis of HCMV infection in the lung differs in these two patient groups (Bower *et al*, 1990, Millar *et al*, 1990).

The introduction of new combination treatment protocols to control HIV infection (highly active antiretroviral therapy or HAART) has led to improved immune functions (Deeks *et al*, 1997). The use of nucleoside analogues and protease inhibitors as part of the new treatment regimen has limited HIV viral load and enhanced $CD4^+$ T cell count.

1.9.4 Congenital infection

HCMV infection is the most common viral congenital infection in humans. It is an important medical and public health problem because it causes damage to the central nervous system. HCMV can be acquired by the fetus either during pregnancy via intrauterine transmission of the virus or perinatally during birth or breastfeeding. The first mode of transmission is associated with more severe complications (Fowler *et al*, 1992). Other significant risk factors for the development of HCMV disease include

the serostatus of the mother, gestational age at the time of transmission, viral load and the particular HCMV strain. Although symptomatic congenital infection and central nervous system (CNS) injury have been reported after recurrent maternal infection, primary maternal infection is more likely to result in symptomatic congenital infection and significant central nervous system implications in infants. Both symptomatic and asymptomatic neonates excrete high titres of virus for long periods of time in the urine and saliva, making them an important reservoir of virus for primary infection in pregnant women. Therefore, day care centres pose a significant risk setting for acquiring primary infection, with virus spread occurring through oral and respiratory routes (Pass and Kinney, 1985, Shen *et al*, 1996). Other important routes of virus transmission include sexual contact and blood transfusion (Demmler *et al*, 1986, Soderberg-Naucler *et al*, 1997). Gestational age is important for the pathogenesis of HCMV infection. Acquisition of virus within the first trimester is associated with more severe sequelae in congenitally infected infants (Anderson *et al*, 1996). *In vitro* studies have implied a role for viral load and type of HCMV strain in the pathogenesis of congenital HCMV infection (Lazzarotto *et al*, 2003). Increased viral load leads to a poorer prognosis in the guinea pig, while different murine CMV isolates exhibit different *in vivo* behaviours (Reynolds 1993, Harrison *et al*, 1995). A comparison of virus loads in various body fluids of the infected infant has shown that detection of HCMV DNA in the cerebrospinal fluid is the most predominant factor for the risk of developing symptoms (Halwachs-Baumann *et al*, 2002).

While 90% of newborns congenitally infected with HCMV are asymptomatic at delivery, 10% will have a variety of symptoms. Abnormalities include thrombocytopenia, haemolytic anemia, elevated hepatic transaminases and cerebrospinal fluid protein, low birth weight, lethargy, hypotonia, periventricular calcification and cortical atrophy (Pass *et al*, 1980). Although some of the symptoms will resolve spontaneously within a few weeks of birth, newborn disease can be severe. Infected infants could develop microcephaly, hearing loss, vision impairment and mental retardation (Ramsay *et al*, 1991, Bale *et al*, 1996, Boppana *et al*, 1996). Unfortunately, between 5% to 17% of infected infants who were asymptomatic at birth will go on to develop hearing loss within the first two years of life (Hanshaw *et al*, 1976).

In conclusion, HCMV is a major pathogen in the immunocompromised host, infecting multiple organs. The pathology in these organs can be caused either directly as a result of viral cytotoxicity or by indirect tissue damage caused by the host immune system, or by both mechanisms (see section 1.9.2). Additionally, HCMV can cause disease in the immunocompetent host (see section 1.9.5).

1.9.5 HCMV and atherosclerosis

Atherosclerosis is a disease of large and medium-sized arteries, characterised by the thickening of the inner portion of the artery wall in association with lipid deposits (Ross *et al*, 1990). Advanced lesions of atherosclerosis can inhibit blood flow, particularly to crucial blood vessels supplying the heart and the brain, and therefore contribute to the pathogenesis of myocardial and cerebral infraction. All blood vessels consist of three layers (tunics), the tunica intima, tunica media and tunica adventitia. The tunica intima is the innermost layer and comprises endothelial cells. The tunica media consists mainly of smooth muscle cells and interspersed proteoglycans, while the tunica adventitia is formed by connective tissue containing elastic and collagenous fibres. The adherence of leukocytes to the endothelium is the first step in the development of atherosclerosis (Masuda and Ross, 1990). The adhered leukocytes then migrate to the subendothelial space and they become filled with lipids, forming activated lipid-filled macrophages, called "foam" cells. The accumulation of "foam" cells leads to the creation of the fatty streak, the first lesion of atherosclerosis. The leukocytes start producing inflammatory cytokines and matrix-degrading enzymes that initiate smooth muscle cell migration. Continued inflammation results in increased numbers of leukocytes migrating from the blood to the lesion, resulting in the formation of fibrous tissue, which further enhances the lesion. The fibrous plaques increase in size and by protruding into the arterial wall they may restrict blood flow. Atherosclerosis is considered to be an inflammatory disease, since its lesions are the result of a continued inflammatory reaction (Ross, 1999).

Epidemiologic data and pathologic studies suggest a link between HCMV infection and atherosclerosis. The search for a viral aetiological agent was initiated after discovering that Marek's disease virus could induce the development of arterial vascular lesions in chickens, similar to those seen in human atherosclerosis (Fabricant

et al, 1978, 1978). The mechanics of atherosclerosis also suggest that an infectious agent, such as a herpesvirus, could lead to endothelial cell injury, exposing the underlying smooth muscle cells and triggering the onset of atherosclerosis (Ross, 1999). In 1987, the first study that implicated a role for HCMV in the development of atherosclerosis took place. Blood samples were analysed from two groups of people; patients who underwent surgery for atherosclerotic vessel disease and a matched control group of patients with high cholesterol levels but no evidence of atherosclerotic disease. The study showed that that 70% of patients undergoing surgery for vascular disease had high levels of HCMV antibodies compared with only 40% of control patients (Adam *et al*, 1987). Two studies in heart transplant patients reported that HCMV infection was significantly associated with the development of post-transplant coronary artery restenosis and graft rejection (Grattan *et al*, 1989, McDonald *et al*, 1989). *In vitro* experiments have shown that CMV infection in the rat model leads to increased smooth muscle cell proliferation even when there was no detectable virus in the vessel wall, suggesting a general proinflammatory effect (Lemstrom *et al*, 1993). All of the above data suggest a possible association between CMV infection and atherosclerosis. However, some other studies show that HCMV is not a major risk factor for the development of atherosclerosis (Hendrix *et al*, 1991, Gulizia *et al*, 1995, Adler *et al*, 1998). Although the role of HCMV remains unclear, it is obvious that the virus is neither necessary nor sufficient for the development of atherosclerosis. Atherosclerosis is a multifactorial disease process and other factors contributing to its development are age, gender and hypercholesterolemia. HCMV could contribute to vascular damage and enhancement of the inflammatory response.

1.9.6 Antiviral therapy

HCMV is an important pathogen and as such, has been and continues to be a target for antiviral therapy. There are three ways in which antiviral therapy can be administered to patients: prophylactically, before the onset of disease, pre-emptively upon virus detection, but prior to the onset of symptoms, or for the treatment of clinically apparent HCMV disease. The most cost effective and ideal administration of antivirals is pre-emptively, avoiding unnecessary exposure to the toxic effects of the drugs. During the last few years, the introduction of sensitive viral detection methods such as PCR and antigenemia assays, have allowed the use of pre-emptive treatment. The antiviral agents currently used for the management of HCMV disease

include the nucleoside analogue ganciclovir (GCV), the pyrophosphate analogue foscarnet and the nucleotide analogue cidofovir. All these drugs have a common target: the viral DNA polymerase. Ganciclovir's mechanism of action relies on its phosphorylation by UL97 viral kinase (Littler *et al*, 1992, Sullivan *et al*, 1992). GCV undergoes further phosphorylation by cellular kinases to its triphosphate form, which acts as a competitive inhibitor of the viral DNA polymerase. There are also many new compounds which are at various stages of development, including tomeglovir and benzamidavir, which act as inhibitors of the viral terminase complex that is involved in cleavage and packaging of viral DNA into capsids (Emery and Hassan-Walker, 2002).

The use of ganciclovir, foscarnet and cidofovir has been proved to be effective in treating GI tract disease and retinitis in AIDS patients (Bowen *et al*, 1996, Spector, 1996, Spector *et al*, 1996, Lalezari 1997, Lalezari *et al*, 1997). However, in the pre-HAART era AIDS patients never regained a fully competent immune system, so long-term therapy was required in order to prevent re-appearance of the original symptoms. This in turn, has led to the development of ganciclovir- and foscarnet-resistant HCMV strains in this patient group (Balfour, 1992, Drew, 1996, Baldanti, 1998). Treatment of solid organ transplant recipients has also been effective in limiting HCMV disease (Cooper, 1991, Duncan and Cook, 1991, Jordan, 1992). More recently, the use of pre-emptive oral ganciclovir therapy in renal transplant recipients during the first twelve weeks post-transplantation, effectively prevented HCMV disease during this time period, and did not result in late onset CMV disease (Sagedal *et al*, unpublished data). In contrast, treatment of bone marrow transplant recipients with HCMV disease, especially pneumonitis, has not been as successful. When used prophylactically, ganciclovir could reduce the incidence of HCMV disease after transplantation, but did not provide a benefit for long-term survival (Goodrich *et al*, 1993, Winston *et al*, 1993). This mortality was caused by non-viral superinfections that resulted from the ganciclovir-induced neutropenia. Pre-emptive treatment has been more effective in reducing the occurrence of HCMV disease in transplant recipients (Egan *et al*, 1998). Despite reducing HCMV levels, antiviral treatment with ganciclovir only, cannot clear HCMV pneumonitis in bone marrow transplant recipients (Schmidt, 1991). The combined use of ganciclovir and HCMV immune globulins has produced better results (Reed *et al*, 1988), although more recent studies have shown that a range of

complications occur later in this patient group due to non-viral superinfections (Reusser, 1996).

1.10 DNA array technology

1.10.1 Genomics and beyond

The form and function of an organism is determined by the expression of genome content. Genome analysis can provide a quantitative measure of gene copy number, as well as the presence of single base differences in the primary DNA sequence. Genome analysis therefore provides a detailed picture of the genetic potential of an organism by defining the precise nucleotide sequence of all the genes and gene regulatory regions. Based on this, a tremendous amount of effort has been directed towards obtaining entire genomic sequences for a number of organisms. Phi X174 bacteriophage was the first organism to have its genome completely sequenced in 1977, bringing virology into the post-genomic era (Sanger *et al*, 1977). Since then, public and private efforts have resulted in complete genome sequences for over 900 species, (Galibert *et al*, 1996, Goffeau *et al*, 1996, The C. Elegans Sequencing Consortium, 1998). Additionally, the completion of the draft sequence of the human genome has been recently announced, predicting 30,000 to 40,000 genes (Lander *et al*, 2001, Venter *et al*, 2001).

Genomic analysis at the DNA level, however, does not provide a measure of gene behaviour and function, as the genome is a relatively static repository of the organism's genetic potential. Therefore, genome sequencing raises the essential task of assigning the role of each gene and deciphering the interactions between genes in that genome. In terms of understanding gene function, knowing the level and timing of gene expression is essential to understanding the biological activity of its encoded protein. Therefore, the collection of genes that are transcribed from genomic DNA (expression profile or "transcriptome") is a major determinant of cellular phenotype, function and ultimately disease. However, transcript levels do not necessarily predict protein function. Nevertheless, proteins can not synthesised without an mRNA and therefore dissecting an expression profile can provide an insight into what type of information a genome is using at any stage. mRNA levels can be very informative

about cell state and activity of the genes, and for most genes, changes in mRNA amount mirror changes in protein levels.

To correctly quantify and interpret the transcriptome, the combination of molecular and computational biology is required. There are a variety of methods used for measuring mRNA abundance, including northern blots, RT-PCR, nuclease protection, cDNA sequencing, cDNA fingerprinting (Ivanova and Belyavsky, 1995, Shimkets *et al*, 1999), subtractive hybridisation (Bonaldo *et al,* 1996, Carulli *et al*, 1998), differential display (Liang and Pardee, 1992), serial analysis of gene expression (SAGE) (Velculescu *et al*, 1995, 2000), rapid analysis of gene expression (RAGE) (Wang *et al*, 1999) and DNA arrays. Although some of these techniques have been employed to measure mRNA levels on a genomic scale (Zhu *et al*, 1997), the DNA array technique has been the most widely used technique for large-scale gene expression studies. HCMV is a complex virus infecting many different cell types, each with its own complex function, resulting in complex disease pathology. Therefore, we need to use post genomic technology to investigate and integrate such complexity. However, in order to interpret the biology systems investigated, they need to be well defined. The post genomic studies need to be forward and hypothesis orientated to aid interpretation. One of the most advanced methods is transcriptome study using DNA microarrays.

Protein synthesis (translation) is the second step in gene expression, and assessing protein activity is also essential for understanding genomic function. The importance of protein-based methods relies on the fact that they measure the final expression product of a gene rather than an intermediate. In some instances, they can also detect post-translational protein modifications and provide information on protein localisation. Protein-based methods include western lots, two-dimensional gels, chromatographic separation and mass spectrometry (O'Farrell, 1975, Gygi *et al*, 1999, Mann, 1999, Oda *et al*, 1999). More recently, protein arrays have been constructed for the measurement of the total protein complement of the cell (proteome) (Lueking *et al*, 1999, Haab *et al*, 2001). However, protein-based approaches are generally more difficult, less accurate quantitatively and have a lower throughput than RNA-based ones. DNA microarrays and proteomics are complementary technologies, and since biology in this millennium will focus on the study of complex biological phenomena,

only their integration will enable us to understand the great complexity lying beneath biological processes.

1.10.2 DNA arrays

DNA arrays are the current development of Southern Blot technology (Southern, 1975). The transition from Southern blots to arrays was made possible by advances in two fields; robotics, which give the ability to automate array construction with reproducibility and accuracy at a microscopic scale, and the use of personal computers that allow safe and accessible storage of vast amounts of data. Like Southern Blots, the principle on which DNA arrays are based is the ability of nucleic acids to hybridise to complementary strands even when present in a complex mixture, forming sequence-specific duplexes. There are 4 main types of array: membrane (or nylon) arrays, Affymetrix GeneChips, Agilent arrays and DNA microarrays (Table 1.4). All DNA arrays consist of DNA sequences (probes) positioned on defined places on a solid support. A solution containing thousands of labelled nucleic acid targets, most often labelled cDNA, is incubated with the array and each immobilised probe specifically hybridises to complementary sequences present in the nucleic acid mix. The intensity of the label at each probe location relates to the abundance of that sequence in the target mixture. In this way, the relative abundance of many specific mRNA species in total cellular mRNA can be measured. Oligonucleotide arrays have been shown to correlate well with SAGE and Northern blots (Harkin *et al*, 1999, Lee *et al*, 1999, Murakami *et al*, 2000). Furthermore, changes in mRNA levels measured by cDNA and PCR product microarrays have also been validated by Northern blots and RT-PCR (Heller *et al*, 1997, Iyer *et al*, 1999).

Array types

Membrane arrays and microarrays are produced by spotting gene-specific PCR-amplified or oligonucleotide DNA onto nylon membrane or glass slides respectively, using robotic deposition. The number of different genes spotted on microarrays varies, but the maximum achieved with current robotic technology is around 39,000 (Cuadras *et al*, 2002). Glass has unique advantages compared to nylon for the production of microarrays. First, glass is non-porous, allowing the use of very small

hybridisation volumes, thus reducing reagent consumption and cost. Biochemical reactions also benefit tremendously from non-porous glass surfaces (Cheung *et al*, 1999). Being impermeable and smooth, it allows rapid diffusion during hybridisation and washing (Southern *et al*, 1999). Second, it is a durable material that can withstand high temperatures and stringent washes. Third, glass can be made optically flat which coupled with its transparency and low fluorescence improve image acquisition and analysis. Finally, two different target nucleic acids can be incubated with the microarray in a single reaction, while nylon arrays are limited to serial or parallel hybridisations. For Affymetrix GeneChips, oligonucleotides are synthesised directly on glass supports (*in situ*) using photolithography. A mercury lamp is used to illuminate through a photolithographic mask on to the surface of the chip, selectively removing photo-labile groups from the growing oligonucleotide chain in a stepwise fashion to create oligonucleotides (Fodor *et al*, 1991, 1993). Current human Affymetrix arrays each contain oligonucleotide probes constituting around 17,000 genes. Each gene is represented by 16-20 different 25-mer oligonucleotides and contains a corresponding mismatch control (with one mismatch relative to the gene sequence) that is used for background subtraction (Lockhart *et al*, 1996). The Agilent system also synthesises oligonucleotides on glass supports, but uses ink-jet technology to deposit reaction components on each spot location (Okamoto *et al*, 2000).

	Filter Array	**Agilent**	**Affymetrix**	**Microarray**
Solid Support	Nylon	Glass	Glass	Glass
Probe	PCR product or oligonucleotide	Oligonucleotide	Oligonucleotide	PCR product or oligonucleotide
Synthesis	Robotic deposition	*In situ* by ink jet nucleotide deposition	*In situ* by photolithography	Robotic deposition
Label	^{33}P	Cy3 and Cy5	Fluorescein	Cy3 and Cy5
Labelling method	Reverse transcription or end-labelling	Reverse transcription or amino-allyl ligation	Reverse transcription or biotinylation	Reverse transcription or amino-allyl ligation
Scanning	Phosphor imager	Fluorescent scanner	Affymetrix scanner	Fluorescent scanner

Table 1.4. Different types of DNA arrays.

Sample labelling and array hybridisation

The nucleic acid mixture hybridised with the array can be derived from cell culture or from tissue and must be of high quality. Sometimes however, the quantity of RNA available is limiting and *in vitro* transcription is employed in order to generate sufficient amounts (amplified RNA or aRNA) (Van Gelder *et al*, 1990, Baugh *et al*, 2001). After obtaining purified total RNA, messenger RNA (mRNA), or aRNA, the sample has to be labelled. This is usually achieved by a single round of reverse transcription, where the RNA of interest is converted to cDNA, with the simultaneous incorporation of labelled nucleotides. The primers used for this are either poly(dT), which primes from the poly(A)-tail of mature mRNA, random primers, or a pool of sequence specific primers. The sample of interest is labelled either radioactively or fluorescently. Fluorescent labels can be detected with either a charge coupled device (CCD) camera or photomultiplier tube (PMT) scanner. Unlike membrane arrays and Affymetrix GeneChips that can analyse one sample at a time, two samples can be hybridised to a microarray or an Agilent array. In two-colour microarrays, sample mRNA is converted to cDNA and is simultaneously labelled with a fluorescent dye. mRNA from a reference RNA source is also labelled with a different dye, the two labelled samples are mixed and hybridised to the array. The most common fluorophores used are Cy3 and Cy5. cDNA can also be labelled after synthesis. Cy3 and Cy5 fluorophores can be coupled to the cDNA through amino-allyl-modified nucleotides. When using Affymetrix GeneChips, RNA can be labelled using a psoralen-biotin derivative or by ligation to an RNA molecule carrying biotin (Wodicka *et al*, 1997). The biotinylated RNA is subsequently labelled by staining with a fluorescent streptavidin construct. Molecules from each sample will bind competitively to the complementary DNA present on the array and this can be measured using a scanner sensitive for the label (Figure 1.6). The outcome of such analyses will be a ratio between the Cy5 and Cy3 signal, representing the relative amounts of each sequence between the two samples. By using a common reference mRNA for a series of microarrays, it is possible to cross-compare all the different gene expression values by comparing the ratio of sample hybridisation signal to reference hybridisation signal across all the arrays.

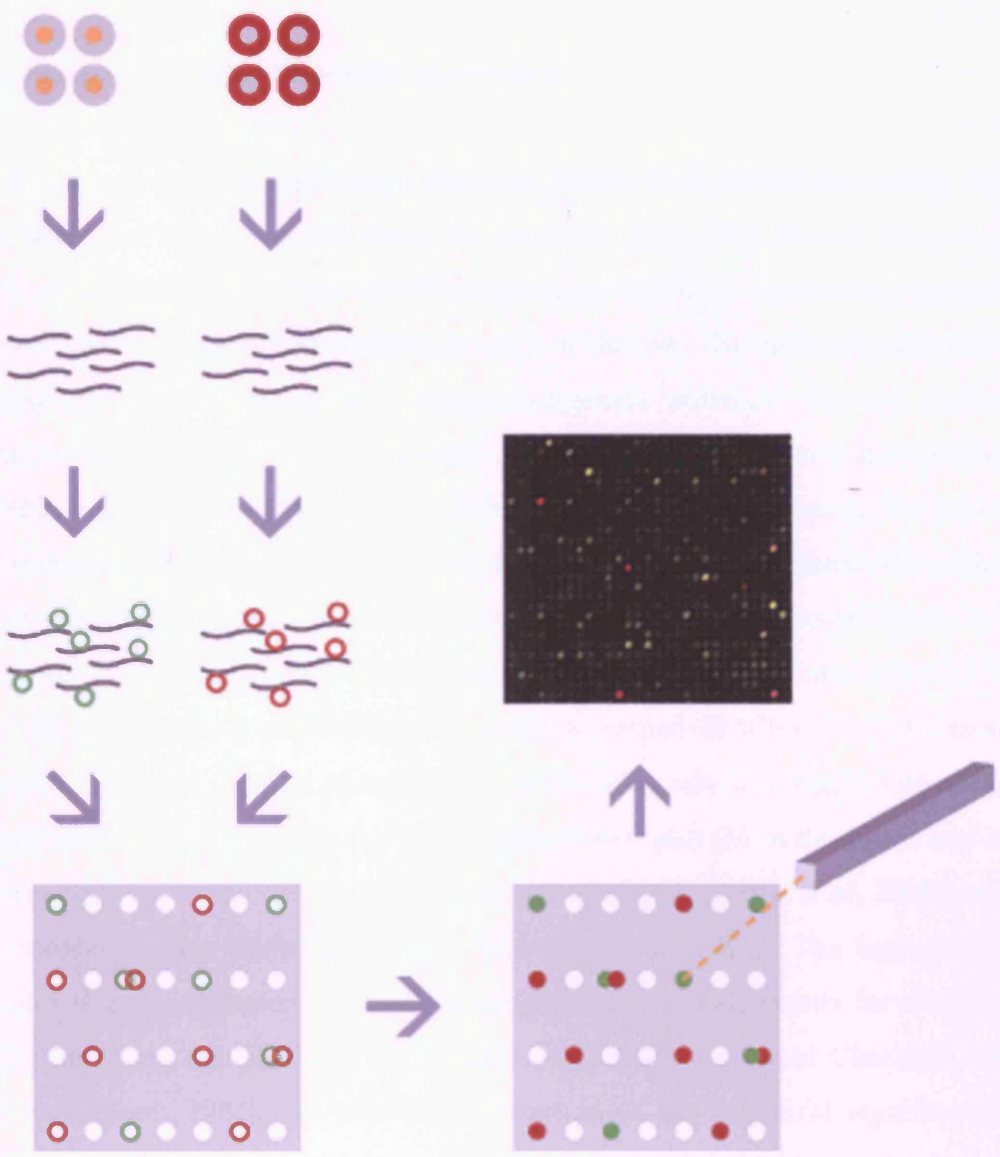


Figure 1.6. Schematic representation of a competitive microarray hybridisation. One RNA sample is labelled with Cy3 (green) and the other with Cy5 (red). These are mixed and hybridised to an array of PCR products or oligonucleotides printed (or synthesised *in situ*) on a glass slide. The hybridised array is scanned with a fluorescent scanner at two wavelengths and an image is generated. The colour of each array element corresponds to the ratio of that sequence between the two samples.

1.11 Microarray data analysis

1.11.1 Analysis of differential gene expression

One of the early steps in analysing expression data is to determine the quality of the entire array and the individual spots on the array. Spots that flawed (ie part of a scratch or badly misshapen) are identified and removed before conducting any further analysis. The fluorescence intensities in each of the two channels are subsequently measured by laser scanning and image recognition software. Several statistical algorithms have been developed to control quality issues in microarray data (Ideker *et al*, 2000, Zhou and Abagyan, 2003). When comparing two samples, for example virus-infected cells and uninfected cells, these are labelled with different fluorophores and are either hybridised on two arrays (when using Affymetrix GeneChips or nylon mambrane) or on the same microarray. Expression ratios are then calculated for each gene and natural log transformed to produce a normal distribution. Differentially expressed genes can then be identified (i) by choosing arbitrary log(2) fold changes (cut-off values), (ii) as outliers in a scatter plot or MA plot (M as the y-axis and A as the x-axis, where M=log ratios and A=average intensities) (Dudoit *et al*, 2001), or (iii) other more complex statistical models (Newton *et al*, 2001). The importance of replicates in array experiments has been emphasised recently, mainly for confirming results, increasing the precision of estimated quantities (Kerr and Churchill, 2001, Yang and Speed, 2002) and allowing estimation of the statistical significance of expression ratios (Thomas *et al*, 2001, Wolfinger *et al*, 2001). Additionally, dye-swap experiments should be performed when using glass microarrays, to account for any biases introduced by dye variation (Jin *et al*, 2001, Tseng *et al*, 2001).

Usually however, the comparison of more than two samples is required. For example, during a time-course experiment, transcript abundance is monitored over time in response to a stimulus. In this case, the samples are hybridised against RNA from untreated cells or mixed reference (Iyer *et al*, 1999). When transcriptional programmes in a number of independent samples are investigated, such as comparing different cellular responses to a common virus, a common reference RNA can be used (Perou *et al*, 1999). The reference RNA is usually a collection of RNAs derived from the samples to be analysed or from related samples that exist in abundance, such as

cell lines. The task of the common reference is to bind to as many of the array elements as possible, in order to obtain expression ratios for all the genes expressed in the samples of interest. Additionally, its use allows minimisation of variations that exist between printed arrays. The experimental sample is usually labelled with Cy5 and the reference sample with Cy3, which are mixed and hybridised onto the arrays. Scanning of the microarray results in the generation of expression ratios for each gene (Cy5/Cy3), which represents the gene expression level in the sample relative to the reference. To make data cross-comparable and remove the reference effect, the data is transformed so that the median expression ratio for each gene across all samples equals 0 (median centering). This data processing enables the expression of each gene in each sample, relative to its median expression level in all samples to be assigned. A number of methods have been suggested for the identification of differentially genes in such experiments. These include methods based on statistical models such as the analysis of variance (ANOVA) model (Wernisch *et al*, 2003) and the mixed effects model (Kerr *et al*, 2000, Wolfinger *et al*, 2001, Park *et al*, 2003). More recently, an associative model has been developed for the identification of differentially expressed genes. This model associates gene expressions presented as residuals in regression analysis against control averaged expressions to a common standard (Dozmorov and Centola, 2003).

1.11.2 Data normalisation

After image processing and before applying the proposed models for analysis, it is necessary to normalise the relative fluorescent intensities in each of the two scanned channels. This is necessary to adjust for differences in labelling and detection efficiencies for the fluorescent labels and for differences in the quantity of starting mRNA from the two samples examined. There are three widely used techniques that can be used to normalise gene expression data, all of which make certain assumptions about the data. These methods are based on the principle that within the data for each experimental array set there is a non-varying set of genes that has the absolute level of gene expression in each sample. This can be a set of genes whose expression levels co-vary across the experimental set ("housekeeping genes" with basic cellular functions whose expression remains constant over different experimental conditions). This method is usually employed for normalisation of data derived from membrane

and Affymetrix arrays (DeRisi *et al*, 1996, Heller *et al*, 1997, Chambers *et al*, 1999). However, this method is potentially flawed in that some studies have shown that for a given subset of "housekeeping genes" their expression level varies in different conditions and cell types (Jenner *et al*, 2001). Alternatively, data can be normalised by adding a group of RNAs to each sample at a defined level for each array (spiked RNAs). For microarrays though, the most common method for normalising the data is "global normalisation", which applies a factor that adjusts the average or median expression ratio to 1. This approach assumes that the majority of genes on the array do not change their expression levels when comparing two mRNA samples (linear regression analysis). It also assumes that the expression ratio is independent of signal intensity. For some microarray experiments the fluorescent intensities however are nonlinear, and local regression techniques are used for normalisation, such as LOWESS (locally weighted scatterplot smoothing) (Yang *et al*, 2002). In any normalisation method, care must be taken in handling genes expressed at low levels, since they can introduce a degree of variation in the system. In fact, the majority of genes with large standard deviation in the level of gene expression occur at low signal intensity. Typically, the final analysis only includes spots where the intensities in the two channels are considerably above background.

1.11.3 Cluster analysis

Once a dataset is normalised, it is important to determine which sets of genes exhibit similar patterns of expression, since generally, genes with similar expression patterns have similar functions. Several distance matrices exist that quantify whether two expression profiles are similar to each other. One is the Pearson correlation, which is a measure of the directions similarity in which two expression vectors point (Heyer *et al*, 1999). A second one is the Euclidean distance, which measures the absolute distance between two points in space. Other measures used include squared Pearson correlation, Spearman rank correlation and mutual information (Butte and Kohane, 2000). Various clustering techniques have been applied to the identification of patterns in gene expression data. Clustering techniques can be classified as agglomerative or divisive, supervised or unsupervised. A description of the various clustering methods follows.

Hierarchical clustering

Hierarchical clustering has become one of the most widely used techniques for the analysis of gene expression data, since it has the advantage that it is simple and the result can be easily visualised. There are various hierarchical clustering algorithms that can be applied to microarray data analysis, including single-, average- and complete-linkage clustering and weighted pair-group average (Michaels *et al*, 1998). Hierarchical clustering is an agglomerative approach, in which single expression profiles are joined together to form groups, which are further joined until the process is complete, forming a single tree (Figure 1.7). The branch lengths of this tree reflect the degree of similarity between items (genes) (Hartigan, 1975, Eisen *et al*, 1998), so shorter branches connecting two genes or samples are indicative of more similar expression patterns. The process of hierarchical clustering starts by converting the data into log(2) expression ratios, with each row representing a single array element (gene) and each column the results from one array (sample). The pair-wise distance matrix for all the genes to be clustered is then calculated and the matrix is searched for the most similar genes or clusters joining them by a node. The distances are calculated between this new cluster and all other clusters, and joining is repeated until all objects are in one cluster. This approach results in the grouping of genes or samples with similar expression patterns. Expression levels are depicted as a colour scale with green representing negative and red positive log(2) expression values. After applying hierarchical clustering, genes with similar function or samples from similar sources usually group together (Perou *et al*, 1999, Ross *et al*, 2000), allowing the assignment of functions for new uncharacterised genes, based on the functions of the genes with which they cluster.

Nevertheless, hierarchical clustering can lead to artefacts. One potential problem is that as clusters grow in size, the expression vector that represents the cluster might not represent any of the genes in this cluster (Quackenbush, 2001). Additionally, if a bad placement is made early in the process, it cannot be corrected (Tamayo *et al*, 1999). Therefore, as the clustering progresses, the gene expression patterns become less relevant. An alternative is to use divisive clustering methods, such as κ-means

clustering or self-organising maps, to segregate data into groups that have similar expression patterns (Kohonen, 1991, Tavazoie *et al*, 1999).

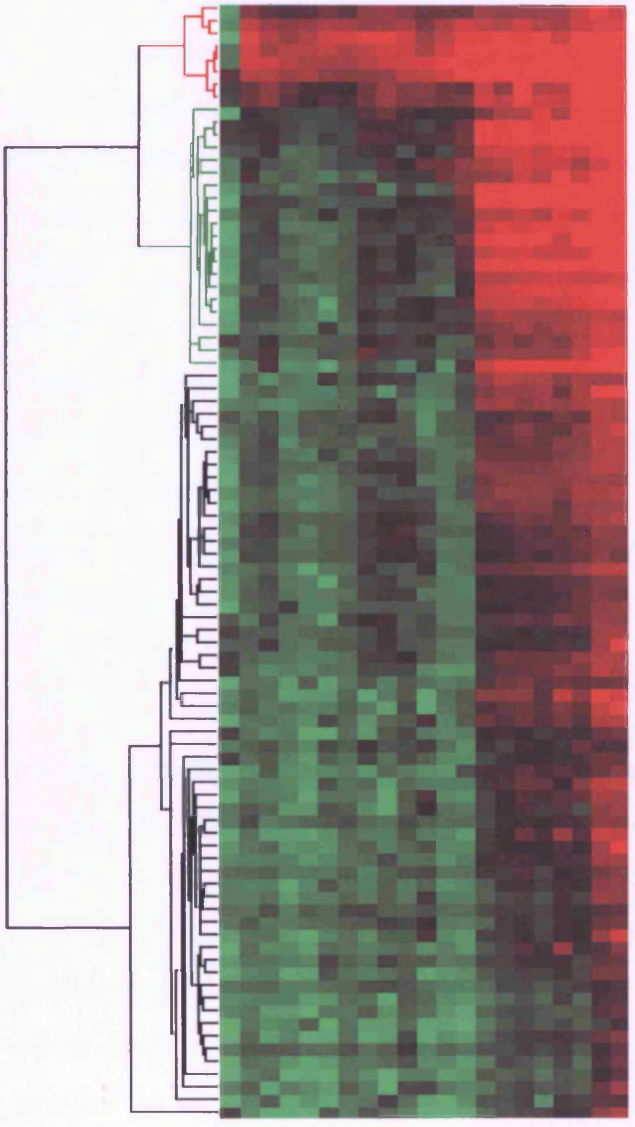


Figure 1.7. Average-linkage hierarchical clustering of gene expression data, during KSHV latency and lytic replication in a B cell lymphoma cell line. Each column represents one array and each row one array element (gene probe). Relative gene expression level is represented as a pseudo-colored representation of log(2) expression ratio with red being above and green below the row/column median level of expression. Groups of genes with related functions cluster together. Figure taken from Jenner *et al,* (2001).

K-means clustering and self-organising maps

While agglomerative techniques usually start with single-member clusters and gradually fuse them together, the divisive methods begin with elements in one large cluster that is broken down into smaller clusters. In κ-means clustering, objects are isolated into a fixed number of clusters (κ), so that the data is more related within a cluster group than between clusters; there is no production of dendrograms (Tavazoie *et al*, 1999). A disadvantage of k-means clustering is that the user needs to decide on the number of divisions in the data. Principal component analysis (PCA or singular value decomposition), is a technique which can be used in combination with κ-means clustering to provide visual estimation of the number of clusters represented in the data. This method reduces high-dimensional data space to two or three dimensions that can be visualised and the axes of the dimensions (x, y, z) are the principal components (Raychaudhuri *et al*, 2000). Another method, which shares features with both agglomerative clustering and κ-means had the ability to remove ill-fitting genes from clusters, which led to its use in studying yeast sporulation data (Zhu and Zhang, 2000).

A self-organising map (SOM) is a neural-network-based divisive clustering algorithm that assigns genes to a series of clusters in 2-dimensional space, on the basis of the similarity of their expression vectors to reference vectors that are defined for each division. The nodes represent the most prominent patterns in the data and similar patterns occur as neighbouring nodes. This method is more structured than κ-means in that the cluster centers are located on a grid. It places genes with similar, but not identical profiles, in neighbouring groups, creating a smooth transition of related profiles. SOMs can be used to order the clusters produced by hierarchical clustering (Chu *et al*, 1998, Eisen *et al*, 1998, Herrero and Dopazo, 2002). As with k-means clustering, the user can use PCA in order to determine the number of clusters that best represents the available data. Overall, the SOM algorithm is an excellent tool for the analysis and visualization of gene expression patterns (Toronen *et al*, 1999).

Supervised clustering

The techniques discussed so far are all unsupervised methods for identifying patterns of gene expression. Supervised methods represent a robust alternative that can be applied if there is existing information about which genes are expected to group together. One example is the support vector machine (SVM) (Brown *et al*, 2000), which finds variables that are most related with a division that can then be used to order a training dataset into known classes. In this way, SVMs can identify genes in the training set that are outliers or that have been previously assigned to the incorrect class, or use biological information to determine expression features that are characteristic of a group. SVMs can also be used for the classification of samples (Furey *et al*, 2000). If gene expression patterns are measured from different patients for whom there is disease-stage classification data, the microarray can be used to create an algorithm, which will be able to categorise other uncharacterised samples. This could lead to the classification of disease, based on molecular expression "fingerprinting". Among others, tree harvesting is another supervised clustering method, which consists of first generating numerous candidate groups by unsupervised hierarchical clustering, and then models the outcome variable as a sum of the average expression profiles of chosen clusters and their products (Hastie *et al*, 2001, Dettling and Buhlmann, 2002). Recently, a tool has been developed which allows the comparison of the results derived using different clustering methods (Sturn *et al*, 2002).

1.12 Applications of arrays

Arrays have found applications in just about every aspect of molecular biology. One of the larger areas of array use is the investigation of pathogen and the integrated host response to the pathogen.

1.12.1 Microbial transcription programmes

One of the main areas for array application is the simultaneous monitoring of thousands of transcripts in different biological settings, leading to identification of new gene networks and an understanding of gene expression patterns. The first

microarray ever, consisted of probes for the model plant *Arabidopsis* (Schena *et al*, 1995). The analysis revealed gene expression differences between root and leaves, implying that this method can be used to evaluate gene expression between two samples. In 1997 it became obvious that the array technology could also help assign functions to uncharacterised genes, since in general, genes with similar expression patterns have similar functions. This conclusion came from work on the yeast *Saccharomyces cerevisiae*, whose entire transcriptional programme was measured using genome-scale arrays (DeRisi *et al*, 1997). More studies have been performed on *Saccharomyces cerevisiae* using different experimental conditions and yeast mutants (Cho *et al*, 1998, Chu *et al*, 1998, Gasch *et al*, 2000, Hughes *et al*, 2000), showing that the time at which a gene is expressed, is associated with its function or cellular response. Since then, the expression of a large number of pathogens has been documented using arrays. Some of these pathogens include *E. Coli* (Richmond *et al*, 1999, Tao *et al*, 1999), *Mycobacterium tuberculosis* (Wilson *et al*, 1999), *Streptococcus pneumoniae* (De Saizieu *et al*, 2000), *Listeria monocytogenes* (Cohen *et al*, 2000), *Salmonella typhimurium* (Eckmann *et al*, 2000, Rosenberger *et al*, 2000) and *Plasmodium falciparum* (Rathod *et al*, 2002). These studies confirm that the physiologic state of bacteria can be inferred from gene expression data. Because genes with similar expression patterns may be regulated by the same regulatory genes, microarray technology can also be used to identify regulatory elements that control the expression network (De Saizieu *et al*, 2000). Gene expression studies may also reveal key regulatory differences that lead to differing virulence between closely related pathogen strains. For example, variations in virulence of *Listeria monocytogenes* serotypes have been correlated with differential transcription profiles of virulence-associated genes (Bohne *et al*, 1996, Sokolovic *et al*, 1996).

1.12.2 Viral transcription programmes

The microarray technology is ideal for studying gene expression of DNA virus genomes. Arrays for a large number of viruses have been constructed, such as human cytomegalovirus (Chambers *et al*, 1999), herpes simplex virus-1 (Stingley *et al*, 2000), MHV-68 (Ahn *et al*, 2002) and KSHV (Jenner *et al*, 2001, Paulose-Murphy *et al*, 2001).

For herpesviruses it is widely accepted that the relative time at which the expression of a gene is detected correlates with the stage of the life cycle in which it is thought to act, therefore knowing a gene's kinetic class can help us determine its function. Herpesviral genes are expressed sequentially in three phases, termed immediate early (IE or α), early (E or β) and late (L or γ), according to the timing of transcription (see section 1.3). The expression of the IE genes is independent of any viral *de novo* protein synthesis. Their products are non-structural proteins, essential for switching on early gene expression. The next set of genes (E genes) encodes products that are involved in DNA replication, DNA repair, and other non-structural proteins. The last genes (L genes) encode mainly structural proteins like capsid, tegument and envelope proteins. Therefore, the study of the whole virus genome can give a coordinated view of the virus's gene expression programme.

The first array analysis for the identification of the kinetic classes of viral genes took place in 1999 by Chambers *et al*, for HCMV (Chambers *et al*, 1999). Before this study, the expression of only one third of the HCMV genome had been documented. Additionally, the results showed 90% agreement with published studies. This analysis allowed the assignment of functions for uncharacterised genes and the search for common promoter elements that may be responsible for the timing of expression. Since then, the genes of HSV-1, KSHV and MHV-68 have also been categorised (Stingley *et al*, 2000, Jenner *et al*, 2001, Ahn *et al*, 2002). In the case of HSV-1, the studies were extended to monitoring viral gene expression patterns in more than one cell type. Additionally, the use of cloned HSV-1 genomes with gene deletions revealed that the immediate-early HSV-1 gene ICP27 is necessary for the expression of a sub-set of early and late genes. Therefore, array technology can help determine the effects of viral genes on the expression of all other viral genes and compare viral gene expression of wild type and mutant viruses. Furthermore, the effects of novel anti-viral drugs on gene expression has been monitored using arrays. DNA array analysis also led to the discovery of viral RNAs on the HCMV capsid, an unexpected finding (Bresnahan and Shenk, 2000).

1.12.3 Understanding of disease

During the last seven years, a number of articles have been published, illustrating the power of microarray analysis in identifying alterations in gene expression levels in human disease. Importantly, gene experession data from array experiments have helped in better understanding of cancer. Among the first microarray studies directly relevant to cancer biology were those of Schena *et al* and DeRisi *et al* (DeRisi *et al*, 1996, Schena *et al*, 1996). Determination of cancer type and stage is often crucial to the assignment of appropriate treatment. One of the first attempts to classify cancer cell lines using microarrays was reported in 1998 (Khan *et al*, 1998). A year later it was shown by another group, that cancer could be classified according to gene expression profile, when comparing two types of leukaemia (Golub *et al*, 1999). This classification was critical, since each cancer type responds differentially to chemotherapy. Since then, classification between multiple cancer classes has been achieved by a number of groups (Perou *et al*, 2000, Khanna *et al*, 2001, Ramaswamy *et al*, 2001, Pomeroy *et al*, 2002). Array analysis has also revealed novel class divisions in B-cell tumour types (Alizadeh *et al*, 2000). There was no general approach for identifying new cancer classes before arrays. Supervised learning has been used to find genes able to predict clinical outcome for a number of cancer types, including embryonal tumours of the CNS, prostate cancer and breast cancer (Pomeroy *et al*, 2002, Singh *et al*, 2002, Van 't Veer *et al*, 2002). Arrays can also be employed to profile drug side effects and correlate gene expression to the sensitivity of tumour cell lines to anti-cancer agents in an attempt to find the targets (Grever *et al*, 1992). Recently, the first report to show global gene expression changes during chemotherapy in a human solid tumor has been documented (Buchholz *et al*, 2002). Microarrays are a useful tool for investigating the mechanisms of drug action. The gene expression signature for the immunosuppressive drug FK506 was studied in *S. Cerevisiae* (Marton *et al*, 1998), revealing new promising targets for the drug. Similarly, microarrays were used to determine the gene expression signature of *M. tuberculosis* exposed to isoniazid (Wilson *et al*, 1999). Analysis of array data identified the induction of the *efp*A gene. This was an attractive drug target, since the gene is only present in pathogenic members of the mycobacterial genus. The analysis of gene expression data can also predict harmful effects. For example, if the

expression profile engendered by a drug candidate is similar to that induced by a known toxic agent, it can be assumed that the drug candidate would also be toxic, a fact that has been confirmed *in vitro* (Braxton and Bedilion, 1998).

Arrays have been utilized to analyse human atherosclerotic lesions from arteria carotis sampled from patients undergoing surgery (McCaffrey *et al*, 2000). One important finding was a strong up-regulation of the early growth response gene Egr-1, a DNA-binding protein that influences the transcription of genes encoding growth factors, cytokines and proteins involved in coagulation. This finding was confirmed by animal experiments and identified Egr-1 as a possible target for therapeutic intervention.

1.12.4 Clinical diagnostics

Arrays have the potential to become an important tool for clinical diagnostics. Once fabrication costs decrease and validated targeted arrays are developed, it will be possible to use them for detection of pathogens in clinical material. Some groups have started investigating the role of microarrays in blood testing and prenatal diagnosis (Dobrowolski *et al*, 1999, Petrik, 2001). More recently, a protein array has been developed for the detection of *Toxoplasma gondii*, rubella virus, HSV types 1 and 2 and cytomegalovirus (Mezzasoma *et al*, 2002), which could be optimised for clinical use.

1.13 Host and pathogen arrays

Viruses are obligate intracellular parasites and as such their relationship with the host plays a key role in their survival. Regulation of infection and *in vivo* pathogenesis involves multiple points of interaction between viral and host factors. Therefore unfolding the cellular and molecular mechanisms underlying these interactions will contribute to our understanding of the processes by which viruses cause disease and provide the underpinning for the development of novel therapeutic strategies.

DNA array technology has the potential to offer an unparalleled view of the transcription changes that underlie the host response to pathogens. Much work has been carried out in this field, monitoring host gene expression upon infection by

viruses of all families except for the ssDNA viruses. The majority of these studies followed host responses after *in vitro* infection either at a selected time point or as a time-course. Zhu *et al.* (Zhu *et al*, 1998) have reported one of the first analyses of the interaction of a human pathogen with a host cell using microarrays. They catalogued 258 genes whose expression levels varied 4-fold early in the HCMV replication cycle, up to 24 hours post infection. Some of these were further confirmed by northern blotting. Since then it has been shown that infection with a variety of viruses dramatically alters host gene expression, modulating the transcription of many genes across a variety of functional groups. (Khodarev *et al*, 1999, Browne *et al*, 2001, Geiss *et al*, 2001, Simmen *et al*, 2001, Cuadras *et al*, 2002, Moses *et al*, 2002). During these time-course experiments, the number of differentially regulated transcripts has been shown to increase with time, probably reflecting increasing numbers of virus genes. The identification of cellular genes, which change in response to infection, may provide essential clues into the pathogenesis of viral infection.

Unfortunately, it is a difficult task to cross-compare results from array experiments in order to identify common patterns of gene expression, due to the use of different cell types and array methods. This however is likely to change, especially after the establishment of MIAME (MInimum standards for the Annotation of Microarray Experiments), a framework for defining the type of data that should be databased and dividing the large amount of data types into defined groups for database operation. This allows microarray labs to design their own databases, which will be compatible with ArrayExpress, the proposed international microarray database. Despite the several issues associated with cross-comparing viral microarray data, some common patterns are apparent, including expression changes in host genes of certain functional groups such as interferon response, cell cycle, protein synthesis and inflammation. These will be discussed in further detail in Chapters 5 and 6.

Microarrays have been used to compare the effects of inactivated viruses with their active counterparts on host gene expression. This analysis can identify differential host responses, dependent on virus gene expression and has been performed for HCMV, influenza virus and HSV-1 (Browne *et al*, 2001, Geiss *et al*, 2001, Mossman *et al*, 2001). The effects of individual virus genes on host gene expression has also been measured by microarrays (Hobbs and DeLuca, 1999, Stingley *et al*, 2000). Some

of these proteins include HCMV IE86 and gB (Simmen *et al*, 2001, Song and Stinski 2002), HIV-1 Tat (De La Fuente *et al*, 2002) and Nef (Simmons *et al*, 2001, Shaheduzzaman *et al*, 2002). DNA arrays have also monitored host gene expression in latently infected cells (Chang and Laimins, 2000, Mikovits *et al*, 2001, Carter *et al*, 2002, Moses *et* al, 2002) and responses to herpesvirus reactivation from latency *in vitro* (Jenner *et al*, 2001, Paulose-Murphy *et al*, 2001, Poole *et al*, 2002). Apart from *in vitro* experiments, gene expression patterns have also been documented after infection *in vivo* (Taylor *et al*, 2000, Bigger *et al*, 2001, Domachowske *et al*, 2002). Additionally, herpesvirus reactivation from latency *ex vivo* (Hill *et al*, 2001, Tsavachidou *et al*, 2001) has been analysed using the gene arrays. Table 1.5 shows human herpesviruses whose effects on host gene expression have been assessed using DNA arrays.

The identification of differentially expressed genes may pinpoint targets for the development of novel therapeutic strategies. HCMV infection of fibroblasts leads to cox-2 up-regulation and prostaglandin E2 release (Zhu *et al*, 1998). When the effect of various cox-2 inhibitors was studied in culture, a dose-dependent inhibition of HCMV growth was observed, suggesting that the virus needs its pathway for its replication (Speir *et al*, 1998, Zhu *et al*, 2002). Since cox-2 inhibitors are already in clinical use, it is possible to test whether they can improve HCMV disease. Also, microarray analysis of KSHV infected DMVECs (Ciufo *et al*, 2001), revealed over-expression of the proto-oncogene c-kit. This up-regulation is essential for KSHV-mediated transformation and a c-kit inhibitor can block proliferation of KSHV-infected cells. Since inhibitors of c-kit signalling are already in clinical use, they could be evaluated for the treatment of Kaposi's sarcoma. The results from these studies suggest that the discovery of potential new targets for treatments can be rapidly achieved using microarray technology.

Family	Species	Array type	Reference(s)
Human Herpesvirus	HSV-1	Membrane	(Khodarev *et al.*, 1999)
		Membrane	(Hobbs and DeLuca, 1999)
		Microarray	(Stingley *et al.*, 2000)
		Microarray	(Mossman *et al.*, 2001)
		Membrane	(Hill *et al.*, 2001)
		Membrane	(Tsavachidou *et al.*, 2001)
	Human cytomegalovirus (HCMV)	Affymetrix	(Zhu *et al.*, 1998)
		Affymetrix	(Browne *et al.*, 2001)
		Microarray	(Simmen *et al.*, 2001)
	HHV-6	Membrane	(Mayne *et al.*, 2001)
	EBV	Membrane	(Carter *et al.*, 2002)
	KSHV	Membrane	(Mikovits *et al.*, 2001)
		Microarray	(Moses *et al.*, 2002)
		Membrane	(Poole *et al.*, 2002)
		Microarray	

Table 1.5. Human herpesviruses whose effects on host gene expression have been assessed using DNA arrays.

HCMV effects on host cells as revealed by array analysis

Infection of fibroblasts by HCMV leads to increased expression of interferon-stimulated genes (Zhu *et al*, 1998), which indicates the activation of the cellular anti-viral response. The number of interferon-responsive genes up-regulated by UV-inactivated HCMV though, is greater than for transcriptionally active virus (Browne *et al*, 2001), suggesting that structural components of the virion trigger the response. Indeed, when cells were treated with purified HCMV gB, most of the cellular genes induced or repressed were also induced or repressed by HCMV infection as well (Simmen *et al*, 2001). This suggested that gB binding is responsible for this up-regulation. HCMV also leads to increased expression of various cytokine mRNAs, such as IL-6 and RANTES. This induction is strengthened by virus inactivation, suggesting that HCMV encodes proteins that block this response (Browne *et al*, 2001). Therefore, as with interferon-responsive genes, cytokine expression can vary according to virus type and the genes expressed. Infection of fibroblasts with HCMV results in the induction of genes involved in the synthesis of prostaglandin E2, such as cyclooxygenase-2 (cox-2) which may act to trigger an immune response against the virus (Zhu *et al*, 1998, Browne *et al*, 2001). HCMV also induces stress-response genes and genes involved in protein synthesis. HCMV affects transcription of a number of cyclins, resulting in inhibition of cell cycle progression. It also changes transcription levels of genes involved in oncogenesis, apoptosis and complement-mediated lysis. These effects will be discussed more extensively in Chapters 5 and 6.

AIMS OF THIS THESIS

The combination of detailed molecular and cellular virology with global views of host and pathogen biological processes by functional genomics, represents an exciting opportunity to delve deeper into the biology of host-pathogen interactions. This PhD therefore aims to use such investigational methods in the context of HCMV. HCMV strains differ in genome content, exhibit different levels of virulence, infect different cell types and different pathologies occur associated with the immune state of the host. To understand HCMV disease we need to start to integrate our knowledge, therefore this thesis aims to:

1. To compare the ability of HCMV strains AD169 and Towne to bind to cells, replicate and mediate cell-to-cell spread of infection, using pair-wise competition experiments in cell culture.

2. To construct a DNA array to examine expression of host and the 19 ORFs found in clinical isolates and low passaged HCMV strains.

3. To describe the transcription program of the 19 ORFs encoded by the UL/b' region during Toledo infection of fibroblasts and endothelial cells.

4. To study the interplay between host and viral gene expression during:
   (i) Toledo infection of fibroblasts and endothelial cells
   (ii) Infection of fibroblasts with AD169 and Toledo

Chapter 2
Materials and Methods

2.1 Cell Culture

2.1.1 Cell culture media

Fibroblast growth medium

Minimal essential medium (MEM) (Gibco Life Technologies) with Earle's salts was supplemented with 200mM L-glutamine, 10,000U penicillin and 10mg streptomycin (Sigma). For the propagation of human embryonic lung fibroblasts, the medium was supplemented with 10% heat-inactivated foetal calf serum (FCS) (Labtech). For the maintenance of cells following virus infection, MEM was supplemented with 3% FCS.

Endothelial cell growth medium

Endothelial cell basal medium was supplemented with 0.5ml human recombinant epidermal growth factor, 2ml human fibroblast growth medium, 0.5ml vascular endothelial growth factor, 0.5ml ascorbic acid, 0.2ml hydrocortisone, 0.5ml human recombinant insulin-like growth factor, 0.5ml heparin, 10ml foetal bovine serum, 0.5ml gentamicin, amphotercin (all from Clonetics).

2.1.2 Cell lines

All cell lines were grown under standard cell culture conditions in a 5%CO_2, 37°C incubator and tested for mycoplasma infection, as described in section 2.15.

Human embryonic lung fibroblasts

Human embryonic lung (HEL) fibroblasts were derived from the lung material of terminated pregnancies. HEL fibroblasts were isolated from lungs of eight to twelve week gestation foetuses. The lung tissue was dissected into small pieces using a scalpel, and digested with 0.1% trypsin / EDTA (Gibco Life Technologies) solution for 30 minutes at 37°C. The universal containing the tissue was then agitated, allowing the large tissue pieces to settle and digestion was repeated. The tissue was

disaggregated with a Pasteur pipette and the cells were centrifuged at 700rpm for 10 minutes and seeded into a 75cm^2 culture flask in MEM supplemented with 10% FCS. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Unless stated otherwise in the text, HEL fibroblasts were used in all experiments.

MRC-5 fibroblasts

MRC-5 fibroblasts are established from normal lung tissue of a 14 week-old male foetus. They were obtained from the ECACC (European collection of cell cultures) collection at passage number 14, maintained in MEM, 10% FCS and passaged in cell culture. They were used for experiments between passage 20 and 30.

Human umbilical vein endothelial cells (HUVEC)

The human umbilical cord was collected in phosphate buffer saline (PBS) and processed within 12 hours. The cord was wiped and cut off from one side to identify the vein. The vein was cannulated and rinsed 3 times by passing PBS through two syringes attached to the cannulae. The cells were then detached from the vein by collagenase digestion. Veins were filled with PBS containing 200U/ml collagenase (Boehringer Mannheim) and incubated for 20 minutes at 37°C. The vein was flushed with 2% FCS in PBS and the collagenase solution collected into a universal. The cells were collected by centrifuging at 1,500rpm for 5 minutes. The cells were re-suspended in growth medium and seeded into a 25cm^2 tissue culture flask that had been coated with 2% gelatine for 1 hour at 37°C. The flask was incubated overnight and fresh growth medium was added. The cells were re-fed every 2 days and passaged when confluent. The endothelial cell growth medium consisted of Iscove's modified Dulbecco's medium (Gibco Life Technologies) supplemented with 20% FCS, 50μg/ml endothelial cell growth factor (Sigma) and 20U/ml heparin. These endothelial cells were used for adaptation and titration of Toledo at passage 2-4. Human umbilical vein endothelial cells used for virus infections, were obtained from Clonetics. These cells were thawed and passaged in culture, using endothelial cell growth medium (Clonetics) and 0.025% trypsin/ 0.01% EDTA solution.

2.1.3 Thawing of cells

Cells were taken from liquid nitrogen and thawed rapidly at 37°C. Cells were re-suspended gently and added to 30 ml of growth medium. The cells were then pelleted at 700rpm for 10 minutes, re-suspended in 30ml of pre-warmed growth media and added to a 75cm^2 tissue culture flask for overnight incubation at 37°C.

2.1.4 Cryopreservation of cells

The cells were trypsinised, re-suspended in MEM, 10% FCS and centrifuged at 700rpm for 10 minutes. The medium was discarded and the cell pellet was re-suspended in 1ml freezing medium. 1ml aliquots were placed into cryovials (Nunc, USA), insulated with tissue, and stored at -70% overnight before being finally transferred to liquid nitrogen.

Freezing medium for fibroblasts: 10% DMSO, 90% FCS
Freezing medium for HUVEC : 10% DMSO, 10% FCS, 80% growth medium

2.1.5 Mycoplasma testing

Cells and virus stocks were negative when tested for mycoplasma. The method used was a nested PCR, with primer sets directed against a highly conserved section of mycoplasma ribosomal DNA, located in the 16S and 23S rRNA genes and the spacer region between (Pruckler and Ades, 1995). The primers used were the following:

5' ACT CCT ACG GGA GGC AGC AGT A 3'	GPO1	First round
5' TGC ACC ATC TGT CAC TCT GTT AAC CTC 3'	MGSO	primers
5' CTT AAA GGA ATT GAC GGG AAC CCG 3'	GPO2	Nested
5' TGC ACC ATC TGT CAC TCT GTT AAC CTC 3'	MGSO	primers

The PCR reaction mix was initially prepared. For 5 samples, the mix contained the following ingredients: 156.5µl H_2O, 25µl 10xPCR buffer, 25µl 2.5mM $MgCl_2$, 16µl dNTPs, 1µl of each primer (1nanomole/µl), and 0.5µl *Taq* DNA polymerase

(AmpliTaq Gold). 45µl of the reaction mix were aliquoted into 0.75ml PCR tubes and overlaid with mineral oil. 5µl of sample (either virus stock or trypsinised cells) were added to the PCR reaction mixture and PCR cycling was performed. Subsequently, 5µl of the PCR product were added to a PCR mix (prepared as before) for a second round of amplification.

The following cycling conditions were performed:

	Round 1	**Round 2**	
Temperature (°C)	**Duration**	**Duration**	**N° cycles**
95	12min	12min	1
94 55 72	30sec 60sec 60sec	15sec 40sec 15sec	40
72	10min	10min	1

The PCR products were analysed by electrophoresis on 3% agarose gels. The expected size of the first round product is approximately 720bp and the second is 145bp.

2.1.6 Viral stocks

The AD169, Towne, ToledoF (fibroblast-adapted) and ToledoE (endothelial cell-adapted) laboratory strains of human cytomegalovirus were a kind gift from Dr. Jane Grundy, Royal Free and University College Medical School, London, UK (MacCormac LP and Grundy JE, 1999). AD169, Towne and ToledoF were propagated by the serial inoculation of cell-free virus on uninfected fibroblasts for 1 hour at 37°C in 5% CO_2, after which the supernatant was removed and the fibroblasts were maintained in medium containing 4% FCS. To prepare the virus-free inoculum, each strain was harvested from the supernatant fluids of infected fibroblasts at days 4 to 7 post infection, clarified by centrifugation at 800g for 20 minutes and stored at -80°C until use. ToledoE (to maintain consistency we called it ToledoE, although it is likely that it is not the prototype Toledo strain) was propagated by the serial inoculation of cell-free virus on uninfected human umbilical vein endothelial cells, by

centrifugation at 1,500g for 1 hour and subsequent incubation at 37 °C in 5% CO_2. Virus-free inoculum was prepared in the same manner for the other strains. AD169, Towne, ToledoF had been highly passaged in fibroblasts for many years, while ToledoE had been initially passaged in fibroblasts and then adapted for growth in endothelial cells.

2.1.7 Viral infection of cell monolayers

35mm dishes or 25cm^2 flasks were seeded with 5x10^5 or 10^6 cells (p4 HUVEC or p27 MRC-5), respectively. These were left in a 5%CO_2, 37°C incubator overnight. The media was removed and the cells washed three times with PBS. The cells were infected with virus diluted in cell culture medium. The flasks were incubated for 1 hour, the inoculum removed and the cells washed three times with PBS. Finally, cell culture medium was added and the cells were harvested at several time-points post infection.

2.1.8 Titration of infectious dose

The virus stock titre was evaluated in slides by DEAFF (detection of early antigen fluorescent foci) test for immediate early/early antigen (IEA/EA) 24 hours post infection. HEL fibroblasts were cultured in 8-well glass slides. The monolayer was infected in duplicate with 250µl of the appropriate virus, in 2-fold dilutions from 10^{-1} to 10^{-4}. The slides were incubated for 1 hour in a 37°C 5% CO_2 incubator. The inoculum was removed and the wells were washed once with medium supplemented with maintenance media. After 24 hours, the culture medium was removed and the monolayer was fixed with 80% cold acetone for 10 minutes at room temperature. The slides were air-dried and tested for IEA/EA positive cells by incubating them with the primary antibody for 30 minutes at 37°C. The slides were washed, air-dried, FITC-labelled goat anti-mouse Ig was added, and incubated for a further 30 minutes at 37°C. After the cells were washed and air-dried, they were mounted with 30% glycerol/PBS solution (Citifluor). The number of antigen forming units (AFU) per ml of each virus stock was calculated, based on the number of IE/E antigen positive cells in two wells. For the experiments described in Chapter 3, virus stocks were titrated by quantitative competitive PCR (QC-PCR) (see section 2.2.1.2).

2.2 Viral fitness experiments

2.2.1 Time-courses of infection

In 1992, a quantitative assay was developed for the detection of HCMV nucleic acid in clinical samples, by using a modified polymerase chain reaction. This technique involved the construction of a control PCR target sequence by PCR mutagenesis to allow the post-amplification quantification of HCMV DNA. The control region was identical to a sequence within the gB coding part of the virus genome, except that a unique restriction site allowed post-amplification differentiation of control/non-control target amplified product (Fox *et al*, 1992).

Viral DNA in culture supernatant relates to viral particle presence. To assess the comparative levels of DNA in cell culture, a time-course experiment using different multiplicities of infection of AD169 on HEL cells at time points 0, 6, 12, 24, 48, 72 and 96 hours was set up. A time-course was also performed using ToledoE in HEL cells and HUVEC. 35mm^2 tissue culture plates were seeded with 5x10^5 cells. Triplicate plates were grown overnight at 37°C in 5% CO_2. The media was removed and the cell sheet washed three times with PBS. Virus was added to the cells and incubated at 37°C in 5% CO_2 for 1 hour. The viral inoculum was removed (time point 0h) and the cells washed three times with PBS, maintenance media was replaced and incubated for the appropriate time period. The cells were harvested for each time point and DNA was extracted. QC-PCR was used to quantitate the levels of HCMV DNA in infected cells.

2.2.1.1 DNA extraction

DNA extraction was performed using the Puregene extraction kit (Flowgen). The media was removed from the cell monolayer and 600µl of the cell lysis solution were added with vigorous pipetting, to ensure complete cell lysis. The lysate was transferred to a 1.5ml Eppendorf tube, 3µl of RNAse were added, the tube was inverted several times and incubated at 37°C for 15 minutes. The lysate was allowed to cool at room temperature, 200µl of protein precipitation solution were added, vortexed vigorously and centrifuged at 13,000g for 3 minutes, leaving the DNA in the

supernatant. This was precipitated with 600µl of 100% isopropanol in a new 1.5ml eppendorf tube by inverting the tube and centrifuged at 13,000g for 1 minute. The DNA was washed twice with 600µl of 70% ethanol, re-suspended in distilled water and stored at -70°C.

2.2.1.2 Quantitative competitive PCR

The PCR reaction mix was initially prepared. For 1 sample (including an extra sample to allow accurate aliquoting), the mix contained the following ingredients: 75.8µl H_2O, 10µl 10xPCR buffer, 2µl 25mM $MgCl_2$ (2mM final), 3µl dNTPs, 1µl gB1 forward primer (100ng/µl), 1µl gB2 reverse primer (100ng/µl), and 0.2µl Taq DNA polymerase (5U/µl). 93µl of the reaction mix were aliquoted to 0.75ml PCR tubes and overlaid with 4 drops of mineral oil. The following primers were used:

QCgb1 5' GAG GAC AAC GAA ATC CTG TTG GGC A 3' (nt 81683-81707)
QCgb2 5' GTC GAC GGT GGA GAT ACT GCT GAG 3 3' (nt 81580-81558)

5µl of sample DNA were added to the PCR reaction mixture. The control plasmid (stock: 10^8 copies/2µl) was diluted with water to $5x10^3$, 10^3 and 10^2copies/2µl. Each control plasmid dilution was then vortexed for ~45 seconds before adding the 2µl of the appropriate dilution to the PCR reaction mixtures. In the samples, positive and negative controls were included. The tubes were placed in the thermocycler-Hybaid omnigene machine.

Cycling conditions consisted of:

Temperature (°C)	Duration	N° cycles
95	15min	1
94 60 72	30sec 30sec 30sec	40
72	10min	1

Agarose gel electrophoresis

A 2% agarose gel was prepared by adding 2g of agarose to 100ml of 1xTBE in a 200ml glass bottle. The agarose solution was boiled in a microwave to dissolve the agarose and 3µl of 10mg/ml ethidium bromide were added to 100ml of the solution. The solution was then allowed to cool to ~50°C. The gel was poured and allowed to set. 3µl of DNA loading dye were added to the wells in a microtitre plate to which 10µl of the PCR reaction would be added. The gel was electrophoresed in 1xTBE buffer at 80mA, visualized under UV light and photographed using Polaroid film.

Restriction enzyme digestion of PCR products

The reaction mixture (for 1 sample) consisted of: 7.5µl H_2O, 2µl 10xbuffer and 0.5µl HpaI. 10µl of the reaction mix were added to 10µl PCR reaction in a 1.5ml microcentrifuge tube, and incubated at 37°C for 2 hours.

Polyacrylamide gel electrophoresis

A 12% acrylamide gel was prepared using the following recipe: 6ml H_2O, 3ml 40% acrylamide solution, 1ml 10xTBE, 75µl 10% APS and 7.5µl TEMED. Each sample was loaded onto the gel and run at 40mA. An ethidium bromide solution was prepared by adding 1.5µl of 10mg/ml EtBr to 100ml of 1xTBE. The gel was placed in solution, stained for 5 minutes and photographed.

10xTBE buffer:	432gr	Tris base	(Sigma)	Diluted in water
	220gr	Boric acid	(Sigma)	
	37.2gr	EDTA	(Sigma)	

2.2.2 Co-infection experiments

Virus stocks were mixed together in known ratios to infect HEL fibroblasts (all experiments performed in triplicate). Cells and supernatants were harvested at several

time-points after absorption and DNA was extracted from cells as described in section 2.2.1.1.

2.2.2.1 Qualitative PCR

PCR was used to amplify a 583-586bp (size difference due to sequence variability between the strains) segment of the CMV gB gene flanking the gp55 cleavage site (Chou *et al*, 1992, 1992). The following primers were used:

5' CTG GGA AGC CTC GGA ACG 3' gB1
5' ACC CAT GAA ACG CGC GGC 3' gB2

Sequences of the gB1 and gB2 primers correspond to nucleotides 1200-1217 and 1765-1782, respectively. The PCR reaction mix was initially prepared. For 1 sample, the mix contained the following ingredients: 77.8µl H_2O, 10µl 10xPCR buffer, 2µl 25mM $MgCl_2$ (2mM final), 3µl dNTPs, 1µl gB1 forward primer (100ng/µl), 1µl gB2 reverse primer (100ng/µl), and 0.2µl Taq DNA polymerase (5U/µl). 95µl of the reaction mix were aliquoted to 0.75ml PCR tubes and overlaid with 4 drops of mineral oil. 5µl of sample DNA were added to the PCR reaction mixture. The tubes were placed in the thermocycler-Hybaid omnigene machine, and the following cycling conditions were applied:

Temperature (°C)	**Duration**	**N° cycles**
95	15min	1
94 60 72	60sec 60sec 2min	39
72	10min	1

The PCR products were checked on agarose gels. When no products were derived from the first round of amplification, a nested PCR was performed. 2µl of the product were added to a PCR reaction mixture (as before) and the same cycling conditions were performed, this time using 20 cycles.

2.2.2.2 Restriction fragment length polymorphism

For restriction endonuclease digest analysis, 9μl of PCR product were incubated with 1μl of MaeIII (Boehringer Mannheim) at 55°C for 2 hours. The resulting DNA fragments were separated on 3% agarose gels and visualized by EtBr staining. The number of DNA base pairs in each fragment for Towne was expected to be 281, 153, and 152; for AD169 was 188, 153, 152, and 90. The ratios of the two viral DNA quantities within cells and cell culture supernatants were estimated using NIH image software (http://rsb.info.nih.gov/nih-image/, Kidd *et al*, 2000).

2.2.2.3 Calculation of viral fitness

The relative fitness (replication rate) differences between the two HCMV variants was calculated according to the formula:

$$S=1/t \ln[q(t)p(0)]/[p(t)q(0)],$$

where q is the proportion of the more fit variant at time 0 and time t, whilst p is the proportion of the less fit variant at time 0 and time t (Goudsmit *et al*, 1996).

2.3 Creation of array probes

2.3.1 PCR amplification

DNA was purified from HEL fibroblasts infected with ToledoE using the Puregene extraction kit, as described in section 2.2.1.1 The concentration of the purified DNA was calculated from the UV absorbance at 260 nm using a UV spectrophotometer.

Primers were designed for the selected HCMV genes (Table 2.1) and PCR was performed for each pair of these primers using a thermal cycler. PCR conditions, such as primer concentration and annealing temperature, were optimised for each primer set.

The PCR reaction mix was initially prepared. For 1 sample, the mix contained the following ingredients: 80.8µl distilled water, 10µl 10xPCR buffer, 2µl 25mM $MgCl_2$ (2mM final), 3µl dNTPs, 1µl of each primer (100ng/µl or 200ng/µl) and 0.2µl Taq DNA polymerase (5U/µl). 98µl of the reaction mix were aliquoted to 0.75ml PCR tubes and overlaid with mineral oil. 2µl of sample DNA were added to the PCR reaction mixture.

The following cycling conditions were used:

Temperature (°C)	**Duration**	**N° cycles**
95	15min	1
94 55 or 60 72	30sec 30sec 30sec	40
72	10min	1

Primer Design Criteria (For Table 2.1): 20-22 bases long
~50% GC content
End in C or G
Avoid AAA....TTT repeats
300-350bp fragment amplified

Gene	PCR product bp	Primer sequences	Primer ng/μl
UL18	197	atagcgagcctcaatg gttagctgtcgggtgatcaggg	100
gB	149	ctgggaagcctcggaacg acccatgaaacgcgcggc	100
UL130	350	tgcggcttctgcttcgtcac gttgcgaccgctcagatacc	200
UL 132	276	ttcatagcggtactgatcgc gatgacgttctcaggatcat	100
UL 133	330	atgggttgcgacgtgcacga gtaacacgctgaacagcagc	200
UL 134	342	accagggaagccagtccggta ccatgccgcagatgccaccc	200
UL 135	334	gagattccaagcaggagaga tatcggcagaccatcctctc	100
UL 136	336	atgtcagtcaagggcgtggag ctcgggataatgacagccgcaa	100
UL 137	260	ttcagcggcagatcgtccat cactgctgccagaatggatg	100
UL 138	278	ctcgtgctgatcgtggccat caacggaccaagatctcgtc	200
UL 139	295	ccactacaggtaccagctcta ggtaacgttgcgtcggaaga	100
UL 140	289	cgctcagactaacgcgacta cgtacgatgacacgctgtta	200
UL 141	258	gcatgcgtctctgtgaaaaa aggagtgtgtcatgaccgtg	200
UL 142	325	cggtcattatccatcgttacc cgcaagtgtgattctcttgcag	100
UL 143	211	gacgattcggattcaacaca aagctgatggacgttggttg	200
UL 144	331	atgaagcctctgataatgctc gatgttggacgcctggagtg	100
UL 145	241	ccatcatgcgtcgtatcacg catcgaggagtcatcgtcgct	200
UL 146	190	tattggcttcatcgcgatcc gtctcggtcctggtgatttc	100
UL 147	247	gcaatcgtcaggaagtgctgg cagcgcagtctgaagtggtg	200
UL 148	244	agatggccttacaggtggag agcaatgtgttgaggtactg	100
UL 149	193	ggtggatcagtgttgctatc gtacgcgtgagtctctgttc	100
UL 150	296	gcgacatgttgcttcgtcta tccttgaacgtctgtggttg	200
UL 151	303	tgctgtggcgtattgttctc ctatcagaaccgccgctatt	100

Table 2.1. Sequences and concentrations of primers used to construct the HCMV-human microarray.

2.3.2 PCR product purification

The PCR products (80µl) were analysed on 1% Nusieve low-melting agarose gel at 100V for 1 hour. The gel was placed under UV light, the DNA bands were excised and put in 1.5ml eppendorf tubes. 3 volumes of Buffer QG (Guanidine thiocyanate, Qiagen) were added to 1 volume of gel and incubated at 50°C for 10 minutes. 1 volume of isopropanol was added and the samples were mixed, applied to a QIAquick column and spun at 13,000rpm for 1 minute. The flow-through was discarded and the column was placed back in the collection tube. 0.5ml of Buffer QG was added to the column and centrifuged again for 1 minute. The flow-through was discarded, 0.75ml of Buffer PE (50% ethanol, 10mM Tris-HCl pH=8.5) was added, the column was left to stand for 5 minutes and centrifuged for 1minute. The flow-through was discarded and centrifugation was repeated. The column was placed into a clean 1.5ml eppendorf tube and 50µl of RNase-free water were added to the centre of the membrane. The column was allowed to stand for 1min, centrifuged twice and discarded. The samples were stored at -20°C. 5µl of the purified PCR products were run on 3% agarose gel.

2.3.3 Cloning

PCR products were cloned into pGEM-T Easy (Promega). A description of the cloning procedure follows.

Optimisation of insert:vector molar ratios

The insert to vector molar ratios were optimised for each product to be ligated, using the following formula:

$$\frac{50\text{ng vector} \times y\text{ kb insert} \times 3}{3\text{kb vector}} = z\text{ ng insert}$$

Ligations

The vector and control insert DNA tubes were briefly centrifuged and the ligation reactions were set-up according to the following table:

Materials	Reaction (µl)	Positive control (µl)	Background control (µl)
2x rapid ligation buffer	5	5	5
Vector (50ng)	1	1	1
PCR product	**x**	-	-
Control insert DNA	-	2	-
T4 DNA ligase	1	1	1
dH_2O (10µl final volume)	**y**	1	3

The reactions were mixed by pipetting and incubated at 4°C overnight.

Transformations

The tubes containing the ligation reactions were briefly centrifuged and 2µl were added to a sterile 1.5ml eppendorf tube on ice. JM109 competent cells (10^8 cfu/µg DNA) were thawed on ice for 5 minutes. 50µl of cells were transferred to each tube containing the ligation reactions and left on ice for 20 minutes. The cells were heat-shocked at 42°C for 45 seconds and returned to ice for 2 minutes. 950µl of room temperature LB broth (20gr LB base/litre dH_2O) were added to the transformed cells and incubated at 37°C (shaking) for 1.5 hours. Two LB agar plates were prepared for each ligation reaction, as follows:

15gr agar/lt LB broth
640µl of 50mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-i-D-galactoside)
2ml of 100mM IPTG (isopropylthiogalactoside)
400µl of 50mg/ml ampicillin

100µl of each reaction were plated and incubated at 37°C overnight. Bacterial colonies containing the PCR product were identified by their white colour due to disruption of the LacZ ORF and therefore the ability to convert X-Gal to its blue colour. They were picked from each plate, added to 5ml of LB broth with ampicillin and incubated at 37°C (shaking) overnight.

Glycerol stocks

850µl of the overnight culture were mixed with 150µl of glycerol and stored at -70°C.

2.3.4 Plasmid DNA purification

1.5ml of overnight culture were transferred to 1.5ml eppendorf tubes and centrifuged at 13,000rpm for 5 minutes. The cells were re-suspended in 250µl of Buffer P1 (50mM glucose, 25mM Tris-HCl pH=8, 10mM EDTA pH=8, 100µg/ml RNaseA). 250µl of Buffer P2 (0.2N NaOH, 1% SDS) were added and the tubes were inverted 4-6 times (Qiagen plasmid DNA preps kit). 350µl of Buffer N3 (3M Guanidine-HCl pH=4.8) were added, inversion was repeated and the tubes were centrifuged for 10 minutes. The supernatants were applied to a column, centrifuged for 1 minute and the flow-through was discarded. 0.5ml of Buffer PB (Guanidine hydrochloride and isopropanol) was added and the flow-through was discarded by centrifugation. 0.75ml of Buffer PE (50% ethanol, 10mM Tris-HCl pH=7) was added, the columns were centrifuged for 1 minute, the flow-through was discarded and centrifugation was repeated for 1 minute. The columns were placed in clean tubes, 50µl of RNase-free water were added and the purified DNA was collected by centrifugation. The samples were stored at -20°C until use.

2.3.5 PCR of purified clones

Clone identities were verified by PCR with the primers originally used to amplify the insert. 2µl of purified plasmid DNA were added to the PCR reaction mix described previously (section 2.3.1), and the following cycling conditions were used:

Temperature (°C)	Duration	N° cycles
95	15min	1
94 60 72	30sec 30sec 30sec	30
72	10min	1

5µl of the PCR products were run on 2% agarose gel.

2.3.6 DNA sequencing

The identity of the clones was also confirmed by DNA sequencing using an automated application of the chain-termination method (Sanger *et al*, 1977). 2µl of

plasmid DNA were added to 8µl of distilled water, the mix was placed at 100°C for 3 minutes and centrifuged. 5µl of this was mixed with 1µl T7 primer (TAATACGACTCACTATAGGG) and 4µl of sequencing mix (containing dATP, dCTP, dTTP, dITP, ddUTP, ddGTP, ddCTP, ddATP and DNA polymerase in Tris buffer, pH 8.9). The PCR mixture was placed in a thermal cycler and subjected to the following conditions:

Temperature (°C)	Duration	N° cycles
96	4min	1
96 50 60	20sec 20sec 4min	30

This sequencing reaction incorporates fluorescent di-deoxynucleotides into daughter DNA strands. DNA was precipitated from the sequencing reaction by adding 2µl sodium acetate (3M), 2µl EDTA (100mM, Sigma) and 1 µl glycogen (20mg/ml, Beckman Coulter) in a final volume of 20 µl, vortexing, and then adding 60µl 100% ethanol (-20°C) and leaving on ice for 10 minutes. DNA was pelleted by centrifugation at 14,000rpm for 15 minutes at room temperature. The supernatant was removed, the pellet washed with 200µl 70% ethanol/water (-20°C), and the tube left open at room temperature until dry. DNA was re-suspended in 40µl de-ionised formamide (JT Baker) for 5 minutes and transferred to a 96-well plate. The sequences were determined using an automated capillary DNA sequencer (Beckman Coulter) following the manufacturer's instructions.

2.3.7 PCR of cloned DNA for arrays

The clones were amplified using common pGEM-T Easy vector primers. These primers anneal at the terminal base before the TA cloning site:

pGEM 5' GCATGGCGGCCGCGGGAATT
PGEM 3' GGCGGCCGCGAATTCACTAG

Similarly, sequences from cellular genes and KSHV were amplified, to use as positive and negative hybridisation controls. Probes were amplified from a 1:40 dilution of purified plasmid DNA in distilled water by PCR in 96-well plates. The constituents of each individual PCR reaction were 77.5µl distilled water, 10µl PCR buffer (20 mM $MgCl_2$), 2µl dNTPs (10 mM), 4µl each primer (100µM), 0.5µl *Taq* DNA polymerase (5U/µl) and 4µl DNA. The cycling conditions used were:

Temperature (°C)	Duration	N° cycles
94	2min	1
94 55 72	30sec 30sec 30sec	40
72	7min	1

2.3.8 PCR product purification

The PCR products were purified using the QIAquick 96 PCR Purification Kit Protocol (Qiagen). 3 volumes of buffer PM were added to one volume of PCR sample. 200µl of PCR product (for pGEM-T Easy clones this constitutes 10µg DNA) were used. The mixtures were added to the QIAquick plate on a vacuum manifold and vacuum was applied. After all liquid was pulled through, the vacuum was switched off, the wells were washed with 900µl of buffer PE and vacuum-dried for 10 minutes. Washing was repeated. Maximum vacuum was applied for an additional 10 minutes to dry the membrane. To elute the DNA, 100µl of buffer EB were added to the centre of each well and vacuum was applied for 5min. 100µl of purified DNA was precipitated with 30µl ammonium acetate (8M) and 125µl isopropanol (-20°C). This was left on ice for 2 hours and subsequently centrifuged at 2,300g for 1 hour at 4°C to pellet the DNA. The plates were inverted to remove supernatant and the DNA pellets washed with 100µl 80% ethanol (-20°C) and centrifuged again at 2,300g for 30 minutes at 4°C. The plates were inverted once more and all supernatant removed by spinning the plates upside down at 20g for 1 minute. Each purified PCR product was re-suspended in 5µl of distilled water (2µg/µl) for printing on glass to create the HCMV-human microarray.

2.3.9 Array printing

HCMV probe DNA sequences along with 5428 probes for human genes (Clark *et al*, 2002), were spotted onto glass slides (Nunc, USA). This was performed by Nicola Cattini at the Institute of Cancer Research (Sutton, UK), using a Flexis gridder (Genomic Solutions, UK).

2.4 Time-courses of infection

Two time-course experiments using 0.01AFU/cell (antigen forming units/cell) of ToledoE in fibroblasts and HUVEC at time points 0 (time of inoculum removal), 6, 12, 24, 48, 72 and 96h were set up. 25cm^2 tissue culture flasks were seeded with 10^6 cells. Duplicate flasks were grown overnight at 37°C in 5% CO_2. The media was removed and the cell sheet washed three times with PBS. Virus was added to the cells and incubated at 37°C in 5% CO_2 for 1 hour. The viral inoculum was removed and the cells washed three times with PBS, replaced with cell culture media and incubated for the appropriate time period. RNA was extracted for each time point, which was used for subsequent hybridisation with microarrays. An aliquot of the RNA was kept to perform RT-PCR, as described in section 2.12. A time-course was also performed using AD169 in MRC-5 cells.

2.5 Total RNA extraction

After the appropriate incubation period the flasks were removed from the incubator and the media discarded. 2.5ml TRIZOL (Gibco LifeTechnologies) were added directly onto the cell sheet, left for 5 minutes and the cell lysate was passed several times through a pipette. 1.25ml aliquots were transferred to RNase-free tubes and stored at -80°C. The frozen lysate was thawed at 37°C in a water bath and centrifuged at 12,000g for 10 minutes at 4°C. The supernatant was transferred to a new tube and incubated at room temperature for 5 minutes before 250µl chloroform were added and the tube shaken for 15 seconds. The tube was further incubated for 3 minutes at room temperature and centrifuged at 12,000g for 15 minutes (4°C). The aqueous phase was transferred to a new tube, and the lower phenol phase containing DNA and protein was stored at -80°C. 625µl chloroform were added to the aqueous phase, the tube shaken for 15 seconds and incubated at room temperature for 3 minutes. The tube was

centrifuged at 12,000g for 15 minutes (4°C) and the aqueous phase transferred to a new tube. 625µl isopropanol were added, the tube vortexed and incubated at room temperature for 10 minutes. It was then centrifuged at 12,000g for 15 minutes (4°C). The supernatant was removed and 1ml 75% ethanol was added to the RNA pellet. Vortexing and centrifugation at 7,500g for 5 minutes (4°C) followed. The supernatant was removed and the pellet centrifuged at 13,000rpm for 1 minute at room temperature. The last traces of ethanol were discarded and the RNA pellet was air-dried at room temperature for 5 minutes. Finally, the pellet was re-suspended in 50µl of RNase-free water.

DNaseI treatment of RNA

After RNA extraction, the samples were treated with DNaseI (Promega) to remove contaminating DNA. The following ingredients were added to the RNA:

RNA (1µg/µl)	100µl
DNase buffer	20µl
DNase I (1U/µl),	10µl
Distilled water	70µl

The tubes were incubated at 37°C for 1 hour and terminator solution was added ($1/10^{th}$ total volume) (0.1M EDTA, 1µg/µl glycogen) to stop the reaction.

RNA purification and precipitation

An equal volume of phenol:chloroform:isoamyl alcohol (Sigma) was added to the reaction mix, the tube was vortexed for 10 seconds and centrifuged at 13,000rpm for 10 minutes at room temperature. The top aqueous layer was transferred to a new tube and phenol:chloroform extraction was repeated. The top layer was transferred again and $1/5^{th}$ volume 8M ammonium acetate and 2.5vol. 95%ethanol were added. The tube was incubated at -20°C for 2 hours and centrifuged at 14,000rpm for 30 minutes (4°C). The supernatant was removed, and 200µl of ice-cold 80% ethanol were added to the pellet.

Spinning was repeated as before and the supernatant was removed. The pellet was allowed to dry for 5 minutes, re-suspended in 30µl DEPC-water and stored at -80°C.

RNA quantitation

The RNA was quantified by diluting 2µl of the RNA solution in 498µl DEPC water, and measuring the absorbance at 260nm in a spectrophotometer. The concentration of RNA (µg/ml) was calculated using the following formula:

A_{260nm}x dilution factor x 40(µg/ml)

0.5µg of total RNA was separated electrophoretically on a 1% agarose gel 1µg to verify the integrity of the RNA.

2.6 mRNA purification

mRNA was purified from total RNA extracts using the Oligotex mRNA midi kit (Qiagen). The reaction mixture volumes were made up according to the total amount of RNA that had been extracted per sample. To the 100µl of RNA solution, 150µl DEPC water, 250µl buffer OBB (37^{O}C [20mM Tris-Cl, pH 7.5, 1M NaCl, 2mM EDTA, 0.2% SDS]) and 15µl Oligotex™ (incubated at 37^{O}C [10% w/v suspension in 10mM Tris-Cl pH 7.5, 500mM NaCl, 1mM EDTA, 0.1% SDS, 0.1% NaN_3]) were added. After mixing, the tubes were incubated at 70^{O}C for 3 minutes followed by incubation at room temperature for 20 minutes. The tubes were centrifuged at 13,000rpm for 2 minutes and the supernatant removed. The pellet was re-suspended in 400µl buffer OW2 (10mM Tris-Cl pH 7.5, 150mM NaCl, 1mM EDTA) and the solution transferred onto the top of a spin column. The columns were centrifuged at 13,000rpm for 1 minute at room temperature and the flow through discarded. The Oligotex was re-suspended in 400µl buffer OW2 and the columns centrifuged for a further 1 minute at 13,000rpm. The flow through was discarded and the spin column transferred to a new tube. The Oligotex was re-suspended in 200µl buffer OEB (incubated at 70^{O}C [5mM Tris-Cl pH 7.5]) and the columns spun at 13,000rpm for 1 minute. A further 200µl buffer OEB (70^{O}C) were added and the columns were

centrifuged again. The flow-through containing the mRNA was kept and quantified by measuring the absorbance of 50µl of mRNA solution at 260nm in a spectrophotometer using an RNase-free UVette (Eppendorf). The mRNA samples were concentrated by pipetting 400µl of mRNA onto the top of a Microcon-30 filter in a Microcon 1.5ml tube (Millipore). The filters were centrifuged at 14,000g for 7 minutes at room temperature and transferred inverted into a new tube. They were finally centrifuged at 1,000g for 3 minutes at room temperature to obtain the purified mRNA.

2.7 *In vitro* amplification of RNA

Gene profiling using glass arrays typically requires up to 0.1-1µg of poly(A) RNA to enable enough probe to be synthesized for each hybridisation. However, this causes a limitation for the microarray technology, since it is often impossible to obtain enough RNA from patient blood samples and biopsies. The same problem can be encountered *in vitro*, where a limited amount of cells must sometimes be used in order to achieve a high multiplicity of infection with a low titre virus. Therefore, a technique has been developed to allow amplified RNA to be generated (aRNA) (Van Gelder *et al*, 1990). The procedure consists of reverse transcription with an oligo(dT) primer bearing a T7 promoter and *in vitro* transcription of the resulting DNA with T7 RNA polymerase, generating hundreds of antisense RNA copies of each mRNA in a sample. A description of the method follows (Salunga *et al*, 1999).

The following were mixed in a 0.5ml PCR reaction tube:

Reagent	**Volume (µl)**
Total RNA (1µg)	10
OligodT-T7 primer (0.5µg/µl)	1

OligodT(21)-T7 primer (MWG):
5' TCT AGT CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG GCG T(21) 3' [56-mer]

These were placed at 70°C for 10 minutes and chilled on ice immediately. The tubes were briefly centrifuged and incubated at 42°C for 5 minutes. The first strand master mixed was prepared according to the following table:

Reagent	Volume (µl)
5x first strand buffer (Gibco Life Technologies)	4
0.1 DTT (Gibco Life Technologies)	2
10mM dNTP mix	1
RNasin (Promega)	1
Superscript RT II (Gibco Life Technologies)	1

The mastermix was added to the samples and incubated at 42°C for 1 hour. 1µl was removed for PCR to check whether intact cDNA was successfully obtained. The following PCR mastermix was prepared:

Reagent	Volume (µl)
10xPCR buffer (Qiagen)	5
10mM dNTPs (Promega)	1
Forward primer (100ng/µl)	2
Reverse primer (100ng/µl)	2
Taq DNA polymerase (5 U) (Qiagen)	0.5
Sterile water	38.5

The mix was subjected to the following cycling conditions and the PCR products were run on agarose gels.

Temperature (°C)	Duration	N° cycles
95	2min	1
95 54 72	30sec 30sec 2min	40
72	7min	1

The following second strand master mix was prepared, added to the remainder (19µl) of the first strand reaction and placed at 16°C for 2 hours.

Reagent	Volume (µl)
Second strand synthesis buffer (Gibco Life Technologies)	30
10mM dNTPs	3
DNA polI (Gibco Life Technologies)	4
E.Coli RNaseH (Gibco Life Technologies)	1
E.Coli DNA ligase (Gibco Life Technologies)	1
DEPC water	92

2µl of T4 DNA polymerase (Gibco Life Technologies) were added and followed by a further incubation at 16°C for 10 minutes. The samples were extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) (Sigma) and the aqueous layer was collected. The aqueous layer was loaded onto a Microcon-100 column (Millipore) and the volume in the column was brought up to 500µl with DEPC water. This was centrifuged at 500g for ~14 minutes and two more rinses were performed. The column was inverted into a clean 1.5ml tube and the sample was collected by centrifugation at 1,000g. The sample volume was reduced to 8µl by vacuum centrifugation.

In vitro transcription (IVT) and purification

The Ampliscribe T7 transcription kit (Cambio) was used for the *in vitro* transcription. The transcription mix was prepared adding the reagents in the following order:

Reagent	**Volume (µl)**
10xAmp T7 buffer	2
ATP	1.5
CTP	1.5
GTP	1.5
UTP	1.5
0.1M DTT	2

These were added to the 8µl of the ds cDNA along with 2µl of T7 RNA polymerase and the mixtures were incubated at 42°C for 3 hours. 1µl of RNase-free DNase was added and placed at 37°C for 15 minutes. The aRNA was purified as before and quantitated using the RNA 6000 Nano Assay (Agilent Technologies), according to the manufacturer's instructions. In some instances two rounds of amplifications were performed to obtain enough aRNA for microarray hybridisations. A description of the generation of second round aRNA follows. Initially 1µl of random hexamers was added (Pharmacia) to the aRNA, the mixture was then heated at 70°C for 10 minutes, chilled on ice and centrifuged briefly. The sample was allowed to equilibrate at room temperature for 10 minutes. The first strand master mix was prepared according to the following table:

Reagent	Volume (µl)
5x first strand buffer (Gibco Life Technologies)	4
0.1 DTT (Gibco Life Technologies)	2
10mM dNTP mix	1
RNasin (Promega)	1
Superscript RT II (Gibco Life Technologies)	1

The mastermix was added to the samples and incubated at 37°C for 1hour to generate a cDNA template. 1µl of RNaseH was added and further incubation at 37°C for 20 minutes followed, to remove RNA from the RNA-cDNA hybrids. The hybrids were denatured by incubation at 95°C for 2 minutes and subsequently chilled on ice. 1µl of the oligo(dT) primer was added and placed at 70°C for 5 minutes, followed by incubation at 42°C for 10 minutes to enable primer annealing, then chilling on ice and brief centrifugation. Double stranded cDNA generation, followed by *in vitro* transcription were performed as described above, resulting in the second round of aRNA.

2.8 Labelling

The isolated RNA, either mRNA or cDNA, was labelled using the CyScribe first strand cDNA labelling kit (Amersham Pharmacia Biotech). The following reagents were added to 5µg of amplified RNA (or 500ng of mRNA) in a 0.5ml PCR tube on ice: 1µl random nonamers, 1µl anchored oligo(dT) and water to a final volume of 11µl. The reaction mixture was incubated at 70^{O}C for 5 minutes in a thermal cycler followed by incubation at room temperature for 10 minutes. Subsequently, 4µl 5x CyScript buffer, 2µl 0.1M DTT, 1µl dCTP nucleotide mix, 1µl dCTP CyDye-labelled nucleotide (Amersham Pharmacia Biotech) and 1µl CyScript reverse transcriptase were added to the reaction mixtures. The Cy3 labelled nucleotide was used for control RNA labelling and the Cy5 labelled nucleotide was used for sample RNA labelling. The reaction mixtures were vortexed, centrifuged for 30 seconds at maximum speed in a bench top microcentrifuge and finally incubated at 42^{O}C for 1.5 hours in a thermal cycler. The RNA was denatured by adding 2.5µl 0.5M EDTA (pH 8.0) and 10µl 0.1M NaOH. The reactions were incubated at 70^{O}C for 10 minutes in a thermal cycler. To neutralise the reaction mix, the following reagents were added: 10µl 0.1M HCl, 3µl CoT-1 DNA (Gibco Life Technologies), and 450µl TE (pH 8.0). 20µl of the

mixture was taken for electrophoresis and the remainder was transferred to the top of a Microcon filter. The filter was centrifuged at 14,000g for 6 minutes at room temperature. 20µl of the filtrate was taken for electrophoresis, the filter transferred to a new tube and 300µl TE added. The tubes were centrifuged at 14,000g for 6 minutes at room temperature. 20µl of the filtrate was taken for electrophoresis, the filter was transferred again to a new tube and 300µl TE added. The tubes were centrifuged at 14,000g for 6 minutes at room temperature. 20µl of the filtrate was taken for electrophoresis and the filter was inverted and transferred to a new tube. The tubes were finally centrifuged at 1,000g for 3 minutes at room temperature. The final volume (less than 12µl) of labelled cDNA was measured and $1/25^{th}$ added to 20µl DEPC water for electrophoresis. The filtrate samples (20µl each) were run on a 1% agarose gel (no ethidium bromide), at 50V for approximately 2 hours. The 20µl samples were loaded in 2µl Orange G loading dye, which is non-fluorescent. The gel was visualised using the Storm 860 phospor-screen scanner (Molecular Dynamics) and the compatible ImageQuant computer software.

2.9 Hybridisation

The Cy3 and Cy5 labelled probes were made up to 14µl with TE (pH 8.0) and the hybridisation mixtures were made as follows: 12µl 20x SSPE (Sigma), 1.1µl 0.5M EDTA, 2µl poly $dA_{40\text{-}60}$ (Amersham Pharmacia Biotech), 2µl yeast tRNA (Sigma), 14µl Cy3 probe, 14µl Cy5 probe, and finally 1µl 10% SDS. The mixture was vortexed and incubated at $98^{O}C$ for 2 minutes followed by incubation at $37^{O}C$ for 20 minutes. After incubation, 1µl 100x Denhardt's solution (Sigma) was added to the probe mixture, and the mix centrifuged at 13,000rpm for 15 minutes at room temperature. The arrays were handled with forceps and only held by the very edges to avoid damage to the area of the slide containing the DNA. The arrays were denatured by immersing them in Milli-Q distilled water ($95^{O}C$) for 2 minutes, and then in 95% ethanol for a further 2 minutes. The arrays were dried by centrifugation in a clean, empty 50ml Falcon tube at 1000rpm for 2 minutes at room temperature. The denatured arrays were transferred to the pre-heated hybridisation chambers, which were kept at $65^{O}C$ on top of a hot block. The arrays were placed in the chambers with the DNA side facing upwards and were heated for 15 minutes on the hot block. The cover slips were cleaned, by immersing them in 95% ethanol for 2 minutes. They

were then dried by centrifugation at 1,000rpm for 2 minutes. The probe (46µl) was quickly transferred to the top of the array (on top of a 65^{O}C hot block) in the chamber and the cover slip was quickly and carefully placed over the probe. 150µl 4xSSPE (65°C) were added to the hybridisation chamber next to the array, and the chamber lid placed on and secured in place using screws. The chamber was quickly transferred to a 65^{O}C water bath and incubated overnight.

The array chamber was removed from the 65^{O}C water bath and transferred to the top of a hot block at 65^{O}C for dismantling. The array was carefully removed and immersed in 2xSSPE at 50^{O}C until the cover slip became dissociated from the array. The array was then immersed in 2xSSPE for 2 minutes at room temperature (with rolling). The array was subsequently washed in 1xSSPE for 2 minutes at room temperature followed by 0.1x SSPE for 3 minutes at room temperature. Subsequently, the array was dried by transferring it to a clean 50ml Falcon tube followed by centrifugation at 1,000rpm for 2 minutes.

2.10 Array scanning

The arrays were scanned at 10µm resolution using the GenePx 4000B array scanner (AmershamPharmacia, Axon instruments, Molecular Dynamics) and the images analysed using GenePix Pro 3.0 software. Cy3 and Cy5 fluorophores were simultaneously excited at 532 nm and 635 nm respectively and the resultant omitted light detected with two PMTs. The voltages across the PMTs were adjusted so that the signals from the array elements were balanced (PMT around 900V). The GenePix software combines the data from the two channels to create a single composite image. A spot location and identification template was fitted over the array image using a spot-finding software algorithm. All the elements on each array were checked by eye and the template altered if necessary, for example if array elements were poor quality for any reason they were flagged as bad. Data were extracted from the image by the software using the adjusted template and the normalisation factor (average ratio between signals in the Cy3 and Cy5 channels) calculated automatically by the GenePix software. If this factor was not 1, the PMT voltages were adjusted and the microarray re-scanned. Expression ratios were calculated as the median of the ratios

between the local background-subtracted Cy3 and Cy5 signals on a pixel-by-pixel basis by the software. The data were exported to a spreadsheet created in Excel, named ArrayAnalyser. The median of ratios were filtered to remove flagged array elements and elements for which the signal to background ratio was below 2 in the Cy5 channel or 1.5 in the Cy3 channel.

2.11 Array analysis

The final array data were analysed using Cluster software (Eisen *et al*, 1998). The program Cluster assembles a set of items (genes or arrays) into a tree, where items are joined by branches proportional to the distance measure between the element pairs (short branches, small distances; large branches, large distances). Array elements for which expression measurements had been filtered from 20% or more of the arrays were removed and the data converted to log base 2. The arrays and genes were median centred (the median expression ratio within each array or of each array element across all arrays was set to 0). A self-organising map algorithm is then applied, in order apply an orientation of nodes generated by hierarchical clustering. Genes and/or arrays were clustered by average-linkage hierarchical clustering and the results were visualised using the Treeview software (Eisen *et al.*, 1998).

Mann-Whitney U (Wilcoxon-rank) test

The Mann-Whitney test is the non-parametric alternative of t-test. The filtered data for 14 arrays were assembled and filtered for genes present in 80% of the arrays and then median centred for both genes and arrays in Cluster as before. The data were then exported to Excel and the expression ratio for each array element converted into ranks relative to that array element across all arrays. A U-value for each array element was calculated by:

$$U_i = n_{i,1}n_{i,2} + ((n_{i,1}(n_{i,1}+1))/2) - R_1$$

Where:

$n_{i,1}$ = the number of filtered expression ratios for the i^{th} array element in infected fibroblasts,

$n_{i,2}$ = the number of filtered expression ratios for the i^{th} array element in infected endothelial cells,

R_1 = sum of ranks for the i^{th} array element in the infected cells.

The U values were converted to the standard normal variable for the i^{th} array element (Z_i) with the equation:

$$Z_i = \frac{U_i - ((n_{i,1}n_{i,2})/2)}{\sqrt{(n_{i,1}n_{i,2}(n_{i,1}+n_{i,2}+1)/12)}}$$

The probability associated with each Z-value was calculated in Excel (NORMSDIST(Z_i)).

2.12 Non-array RT-PCR

RT-PCR was performed using the appropriate primers for each HCMV UL/b'gene, to verify results from microarray experiments. The reverse transcription reaction using the GeneAmp PCR kit (Perkin Elmer) contained: 2µl 10xPCR buffer, 2.8µl $MgCl_2$, 2µl of each deoxynucleoside triphosphate, 1µl RNase inhibitor, 1µl random hexamers, 1µl reverse transcriptase and 1µg RNA sample, made up to 20µl with RNase-free water. Following the reverse transcription step, PCR was performed for the genes using the appropriate primers. Each reaction mix contained 5µl 10xPCR buffer, 1µl 10mM dNTPs, 200ng of each primer, 2.5U Taq DNA polymerase and 1µl of cDNA made up to 50µl with sterile distilled water. The thermal cycling conditions consisted of:

Temperature (°C)	Duration	N° cycles
95	15min	1
94 60 72	30sec 30sec 30sec	40
72	10min	1

The PCR products were analysed on 1% agarose gel and visualised using ethidium bromide under UV transillumination.

Chapter 3
Fitness differences between AD169, Towne and Toledo

3.1 Introduction

Traditionally, HCMV detection in a sample has been carried out using tissue culture techniques. However, these techniques are time-consuming and have a low inherent dynamic range. The availability of modern molecular techniques has overcome these problems, enabling us to measure viral load *ex vivo*. Furthermore, quantitative assays have been developed in order to differentiate between active and latent infections in patients, also providing us with information on disease progression and efficiency of antiviral drugs (Gerna *et al*, 1995, 1995). More specifically, in 1992, a quantitative assay was developed for the detection of HCMV nucleic acid in clinical samples, by using a modified polymerase chain reaction. This technique involved the construction of a control PCR target sequence by PCR mutagenesis to allow the post-amplification quantification of HCMV DNA. The control region was identical to a sequence within the gB coding part of the virus genome, except that a unique restriction site allowed post-amplification of control/non-control target amplified product (Fox *et al*, 1992).

The quantification of HCMV DNA has prognostic value and provides clues into the pathogenesis of viral replication (Bowen *et al*, 1995, Cope *et al*, 1997, Mutimer *et al*, 1997, Bowen *et al*, 1998, Gor *et al*, 1998). Additionally, it is a useful tool for fitness determinations of different virus subpopulations in cell culture and *in vivo*, where according to Darwinian competition, replication of mixtures of 2 or more virus strains would be expected to result in the eventual selection of the fittest virus. Relative fitness values, determined by growth competition experiments between two viral populations in cell culture and *in* vivo, can provide insights into the basic features of disease progression (Quinones-Mateu *et al*, 2000). However, the long-term survival probability of a virus includes parameters other than replication capacity, such as adsorption, internalisation and transmissiblility.

Subsequent to initial virus entry and replication, HCMV disseminates to many organs *in vivo*. However, certain organs such as salivary glands secrete higher levels of virus than others and organs show different types of damage (Grundy, 1990). The underlying reasons for these observations are poorly understood. Clinical data have demonstrated that HCMV strains exhibit different levels of virulence depending on their passage history in cell culture (Quinnan *et al*, 1984, Brown *et al*, 1995).

Extensive comparisons of the restriction enzyme profiles of Toledo and a highly passaged variant of Towne revealed an additional region of sequence at the right edge of the Toledo UL component (Cha *et al*, 1996), termed UL/b' region and encoding 19 ORFs. Although these genes are not essential for replication *in vitro*, their maintenance in clinical isolates suggests that the encoded proteins are important for HCMV pathogenesis *in vivo*. The importance of fitness determinations became evident in 1976, when it was proposed that the infecting strain of HCMV is important for clinical outcome (Huang *et al*, 1976), along with the intensity and duration of viral replication. High and low passage HCMV strains exhibit tropism differences *in vitro*, suggesting that different tissue tropism may occur *in vivo*. Indeed, differences in the distribution of HCMV infection in leukocytes from tissues of patients have been demonstrated (Sinzger *et al*, 1996). Furthermore, reports have shown that different clinical isolates obtained from transplant patients had specific tropisms for different cells (Torok-Storb *et al*, 1993), suggesting that differences in tropism might account for differences in pathogenicity.

Apart from phenotypic differences, isolates from different individuals display considerable genomic variation as defined by fingerprinting methods such as restriction fragment length polymorphism (RFLP). HCMV strains have been classified into 4 genotypes based on RFLP of a fragment corresponding to the cleavage site of gB (Chou *et al*, 1991, Chou and Marousek, 1992). gB is an envelope glycoprotein, involved in multiple levels of virus replication such as virus entry as well as cell-to-cell spread and syncytium formation (Navarro *et al* 1993, Tugizov *et al*, 1994, Navarro *et al*, 1997, Meyer-Konig *et al*, 1998, Wang *et al*, 2003). The ease of identifying the gB genotypes by restriction endonuclease polymorphism has facilitated epidemiological studies of the importance of gB genotype with HCMV disease outcome. Although specific gB genotypes have been reported to be associated with different clinical outcomes of HCMV infection (Fries *et al*, 1994, Bongarts *et al*, 1996, Shepp *et al*, 1996, Torok-Storb *et al*, 1997, Trincado *et al*, 2000), there is controversy on the matter, since a number of studies have found no association between gB types and subjects with different risks of developing HCMV disease (Rasmussen *et al*, 1997, Gilbert *et al*, 1999, Arista *et al*, 2003).

The aim of this chapter is to determine the replication dynamics of HCMV strains AD169 and Toledo *in vitro* as well as compare the ability of AD169 and Towne to bind to cells and mediate cell-to-cell spread of infection using pair-wise competition experiments in cell culture.

3.2 Results

3.2.1 HCMV strain identification

In order to confirm the identity of the virus strains used in my experiments, RT-PCR was performed using RNA extracted from fibroblasts infected with either AD169, Towne or ToledoE, using specific primers for gB, UL18, UL132 and selected UL/b' region genes (Figure 3.1). The Towne strain was obtained from the ATCC and has been passaged approximately 135 times in fibroblasts. UL136, UL140, UL148 and UL151 transcripts were detected in late-stage Towne-infected fibroblasts. Published restriction enzyme profiles of Towne between 25 and 32 passages in culture are consistent with the presence of the UL/b' region in the viral DNA (Huang *et al*, 1980). Further support for this conclusion was obtained when a portion of the variants in the Towne stock from the ATCC (passage 152) were shown by hybridisation to contain sequences highly homologous to the novel sequences of the Toledo UL/b' region (Duke *et al*, unpublished data). Therefore, the Towne strain used in my experiments is more similar to wild type HCMV than AD169. I detected UL148 transcripts in late-stage AD169-infected fibroblasts. This was not an unexpected finding, since AD169 contains a region that shows homology to a part of the UL148 gene from Toledo (Figure 3.1).

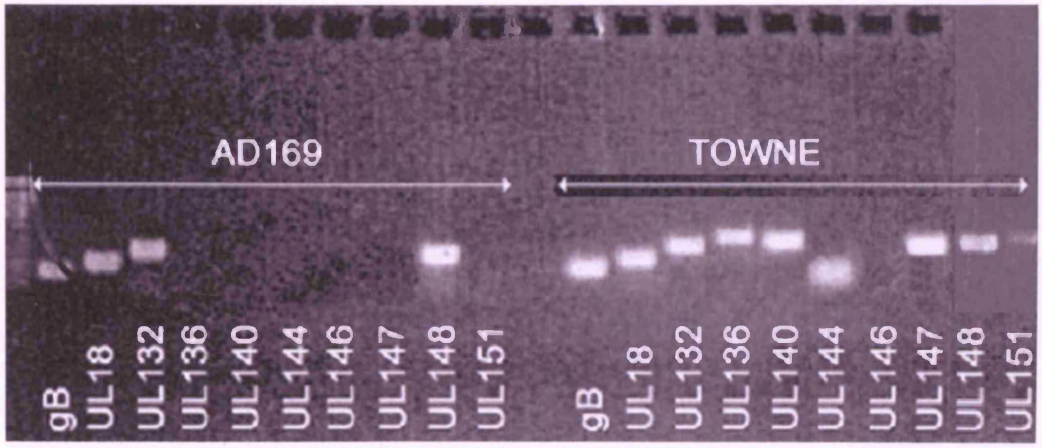


Figure 3.1. RT-PCR using RNA extracted from fibroblasts infected separately with AD169 and Towne.

3.2.2 Time-courses of gB DNA in cells infected with HCMV

Viral DNA in culture supernatant relates to viral particle presence. To assess the comparative levels of gB DNA in cell culture, a time-course experiment using different multiplicities of infection (MOIs) of AD169 in HEL cells at time points 6, 12, 24, 48, 72 and 96 hours was set up. A time-course was also performed using ToledoE in HEL cells and HUVEC. The presence of HCMV DNA was initially confirmed using qualitative PCR (Figure 3.2A). The levels of gB DNA were subsequently quantified using QC-PCR (Figure 3.2B), and plotted against time as shown in figures 3.3 and 3.4. The results for the time-course of infection using AD169 at different MOIs indicate that DNA levels rose exponentially for each MOI, up to 96 hours after infection. Furthermore, the levels of gB DNA were dose dependent with the viral inoculum, with increasing levels of DNA detected at increasing multiplicities of infection (Figure 3.3).

Estimation of gB DNA levels in fibroblasts infected with either AD169 or ToledoE showed that AD169 replicated better than ToledoE in fibroblasts, reaching $1.4x10^9$ gB copies/μg DNA, compared to $8x10^7$ gB copies/μg DNA of ToledoE. Assessment of the replication of ToledoE in two different cell types, fibroblasts and HUVEC, indicated that the virus replicates more efficiently in fibroblasts, as determined by peak virus production after 96 hours ($8x10^7$ and $1.9x10^7$ gB copies/μg DNA, respectively) (Figure 3.4). Additionally, the mean doubling times of AD169 and Toledo were calculated over a 72-hour period (Table 3.1). The doubling times in fibroblasts were 6.5 and 6.9 hours for each strain, respectively. The doubling time of Toledo in HUVEC was 7.3 hours (Table 3.1), indicating that infected fibroblasts and endothelial cells produce virus at the same rate but at varying amounts (in this case, less virus is produced in endothelial cells).

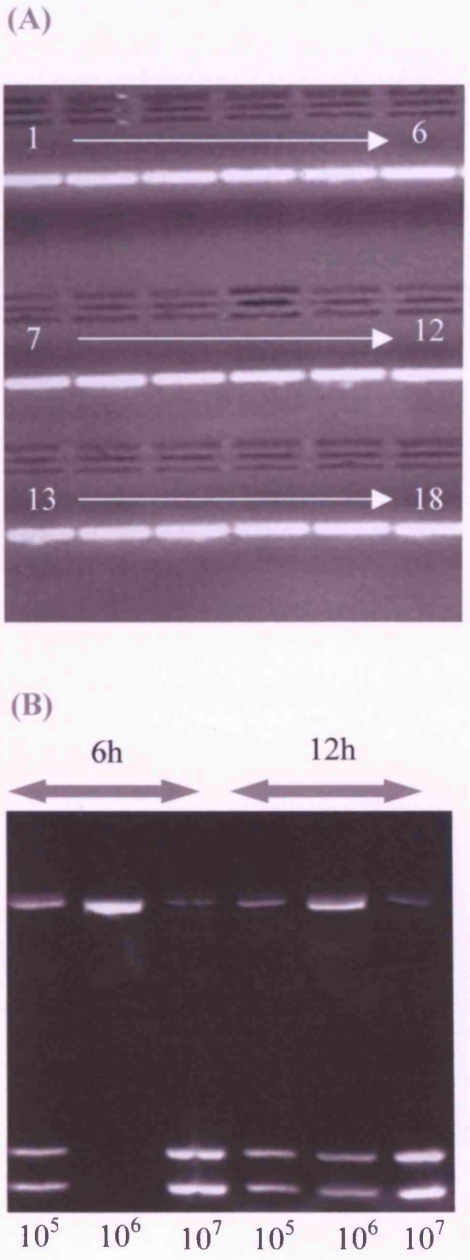


Figure 3.2. Figure showing **(A)** qualitative gB PCR results for HCMV-infected cells and **(B)** an example of quantitative PCR using DNA extracted from fibroblasts infected with AD169.

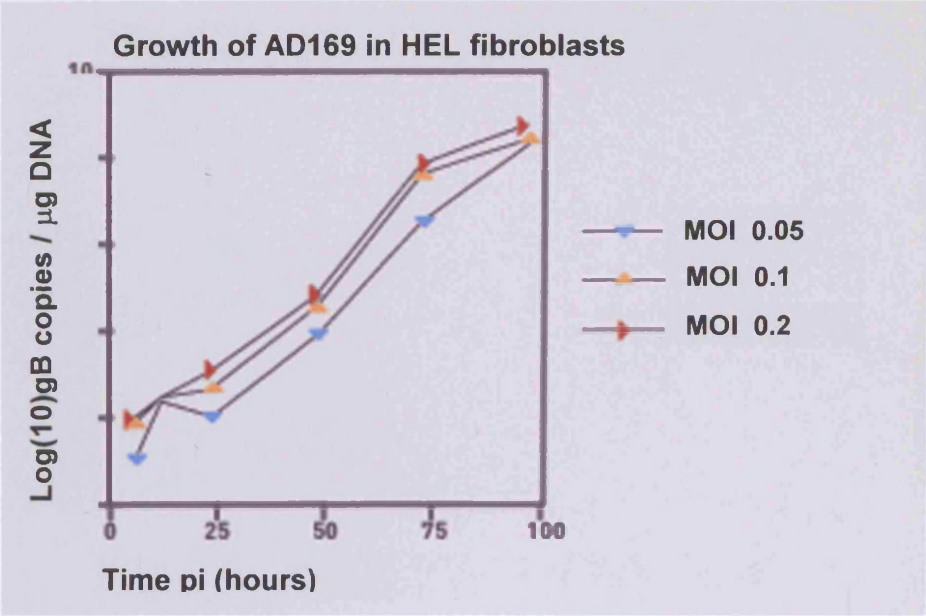


Figure 3.3. Levels of HCMV gB DNA produced during an *in vitro* time-course of HEL cells infected with AD169 at different MOIs.

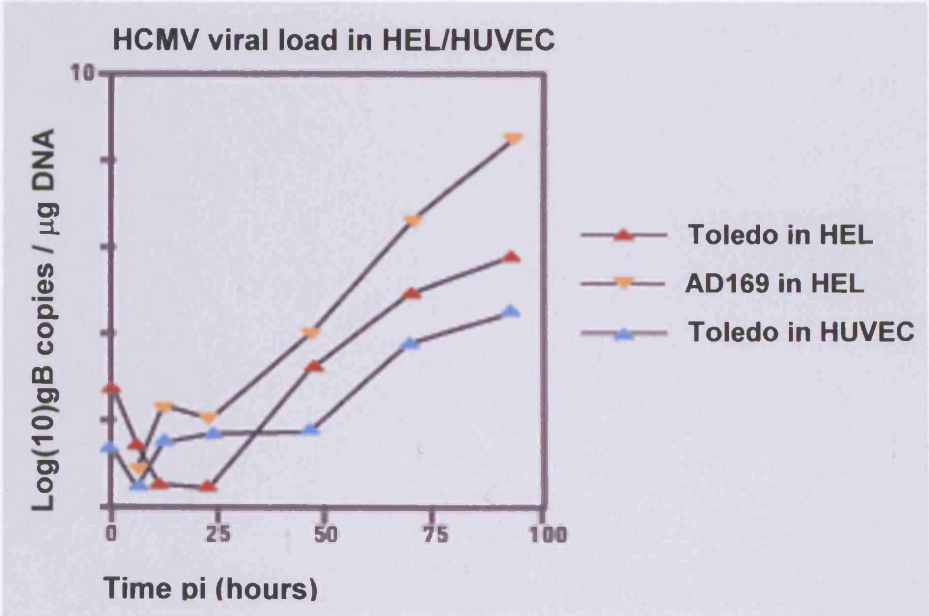


Figure 3.4. Levels of HCMV gB DNA produced during an *in vitro* time-course of HEL cells or HUVEC infected with ToledoE.

Time pi (h)	AD169 in HEL (gB/μg DNA)	ToledoE in HEL	ToledoE in HUVEC
inoculum	$2x10^6$	$3x10^6$	$3x10^6$
6	$3.2x10^5$	$5.7x10^5$	$2x10^5$
12	$1.5x10^6$	$2.2x10^5$	$6.3x10^5$
24	10^6	$1.9x10^5$	$8x10^5$
48	$0.9x10^7$	$4x10^6$	$8.5x10^5$
72	$1.7x10^8$	$3x10^7$	$8.3x10^6$
96	$1.4x10^9$	$8x10^7$	$1.9x10^7$
	$t_{1/2}$ = 6.5h	**$t_{1/2}$= 6.9h**	**$t_{1/2}$=7.3h**

Table 3.1. Table showing the levels of HCMV DNA and doubling times in infected cells.

3.2.3 Assessment of ToledoE infectivity in different cell types

The level of ToledoE infection in fibroblasts and endothelial cells was evaluated by staining for HCMV immediate early and early (IE/E) antigens with specific antibodies. The percentage of infection was detected by visual fluorescent microscopic examination of cells on cytospins. 24 hours after virus inoculation, IE/E antigens were detected in 80% and 40% of fibroblasts and HUVEC, respectively. This finding is consistent with previous results showing that ToledoE replicates to higher levels in fibroblasts than endothelial cells (see section 3.2.2). It has been shown that HUVEC are not permissive for AD169, due to inefficient transport of HCMV DNA into the nuclei of infected HUVEC (Slobbe-van Drunen *et al*, 1998). The slightly reduced virus entry into HUVEC might explain why even endothelio-tropic HCMV strains like ToledoE, replicate to higher levels in fibroblasts.

(A) **(B)**

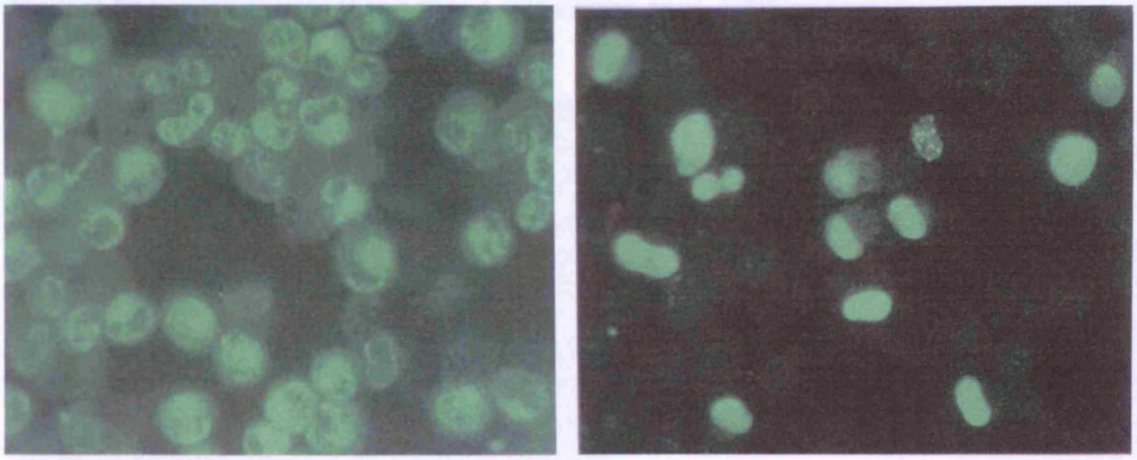


Figure 3.5. Detection of IE/A antigens in (A) fibroblasts and (B) HUVEC infected with ToledoE, using immunofluorescence (detected 24h after inoculation).

3.2.4 Pair-wise competition experiments *in vitro*

3.2.4.1 Cells, viruses and infections

AD169 and Towne virus stocks were titrated by QC-PCR and mixed together in known ratios to infect HEL fibroblasts (all experiments were performed in triplicates). Cells and supernatants were harvested at several time-points after adsorption and DNA was extracted from cells (see section 2.2.1.1). PCR was used to amplify a segment of the HCMV gB gene (583-586bp), flanking the gp55 cleavage site (Chou and Dennison, 1991), containing the major region of sequence variability within this gene (Chou, 1992). For restriction endonuclease digest analysis, PCR products were incubated with MaeIII and the resulting DNA fragments were separated by agarose gel electrophoresis. The number of DNA base pairs in each fragment for Towne was 281, 153, and 152; and for AD169 was 188, 153, 152, and 90 (Figure 3.6). The ratios of the two viral DNA quantities within cells and cell culture supernatants were estimated using NIH image software and the relative viral fitness of the two variants was calculated using the following formula: $S=1/t \ln[q(t)p(0)]/[p(t)q(0)]$, where q is the proportion of the more fit variant at time 0 and time t, whilst p is the proportion of the less fit variant at time 0 and time t (Goudsmit *et al*, 1996).

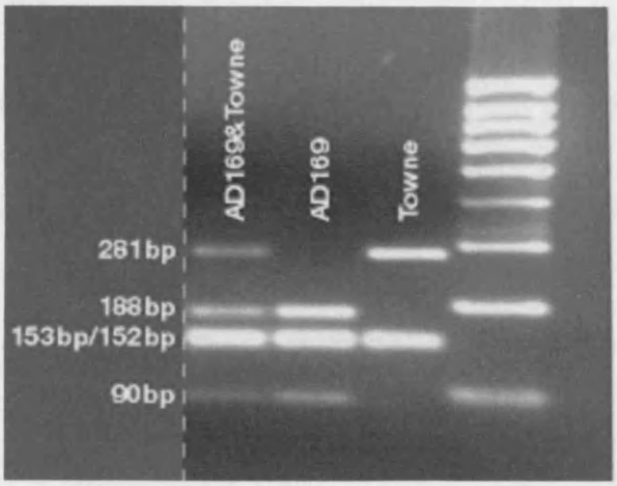


Figure 3.6. HCMV DNA fragments separated on 3% agarose gel after restriction fragment length polymorphism analysis. The number of DNA base pairs in each fragment for Towne is 281, 153, and 152; and for AD169 is 188, 153, 152, and 90.

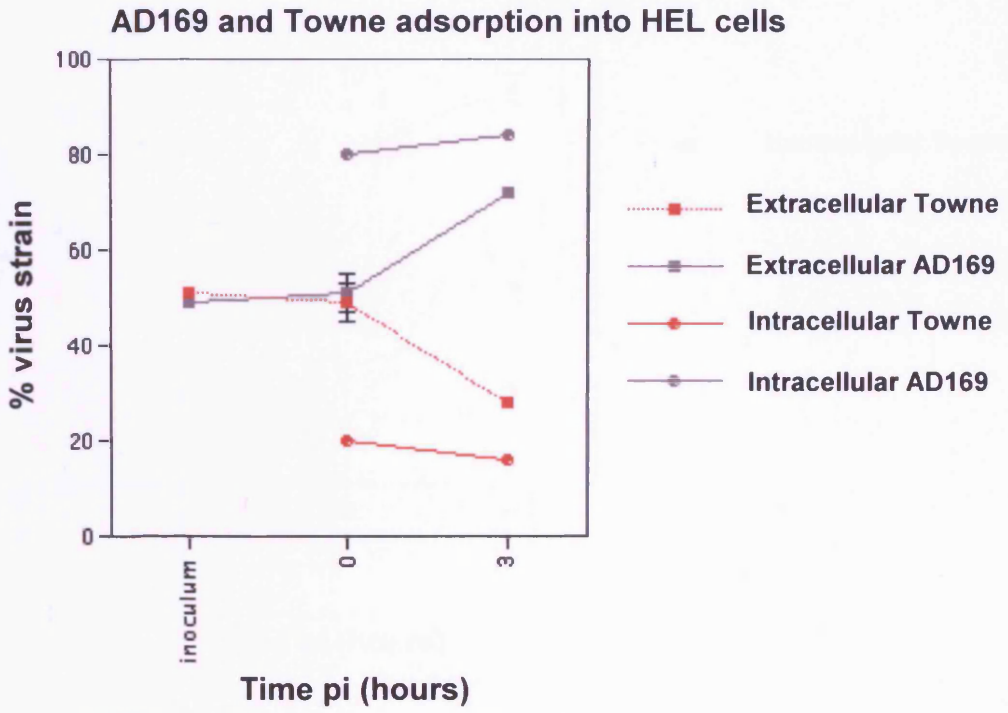
3.2.4.2 Assessment of virus adsorption

Infection is initiated after attachment of viral particles to cells and subsequent internalisation. HCMV infection is likely initiated by the specific binding of the virus, via one or potentially several viral envelope proteins, to one or more cellular structures, although the structures responsible for these processes are still unclear (Kari and Gehrz, 1992, Compton *et al*, 1993, Wang *et al*, 2003). Studies with several viruses have shown that the receptor selected by a virus may facilitate infection and persistence by triggering intracellular events essential for infection. Furthermore, the outcome of infection could also rely on differences in the ability of different strains to bind to cells.

To investigate this, HEL cells were infected with $5x10^7$ genome equivalents of a Towne and AD169 mixture (63/27%, respectively). 1 hour after virus inoculation, the ratio of Towne to AD169 in the supernatant remained virtually unchanged compared to the inoculum, while Towne constituted 57% of the total virus population in the cells. 3 hours after virus adsorption, AD169 became the predominant virus population in the supernatant, while the amount of Towne in the cells increased to 62%. These findings suggest that Towne might be absorbed to cells more efficiently than AD169 (Figure 3.7). The same experiment was repeated using a virus mixture comprised of equal quantities of the two HCMV strains. Although the ratio of Towne to AD169 in the supernatant remained virtually unchanged 1 hour after inoculation, 3 hours later, AD169 comprised 72% of the virus population (Figure 3.8). When comparing the content of intracellular virus at 1 and 3 hours after infection, no significant change in the distribution of the two variants was observed.

To investigate this further, HEL cells were infected with the virus mixture (40% Towne, 60% AD169) at 4°C, allowing virus adsorption but minimising virus entry. 3 hours after adsorption, Towne comprised 33% of the total virus population bound to cells, while only 17% of the total extracellular virus (Figure 3.9). The cells were subsequently transferred to 37°C to facilitate virus entry.

(A)



(B)

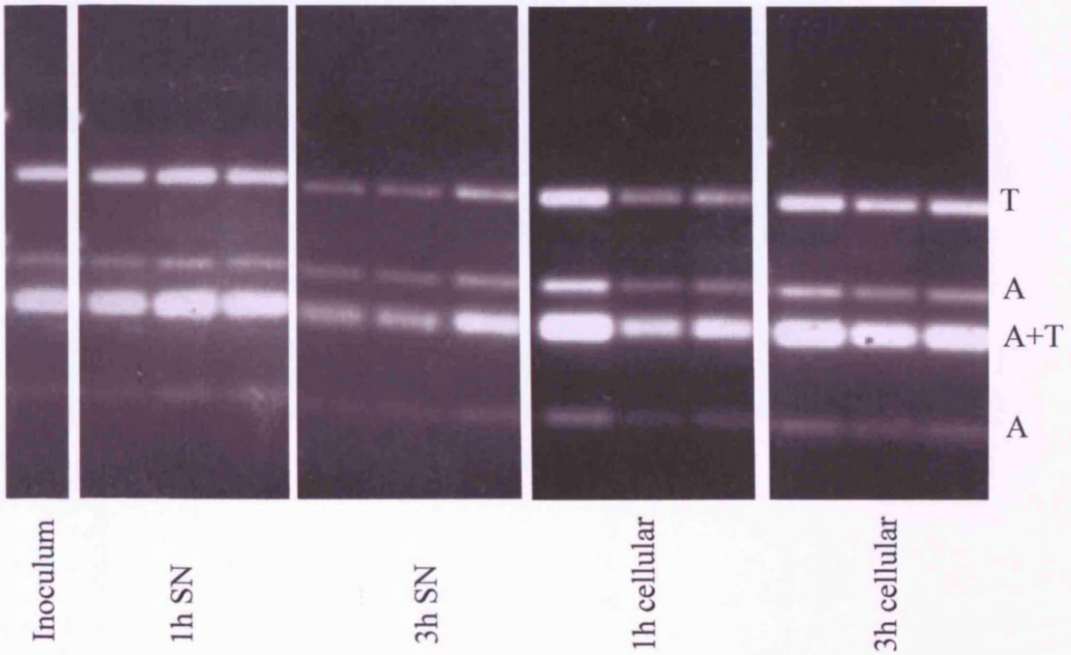
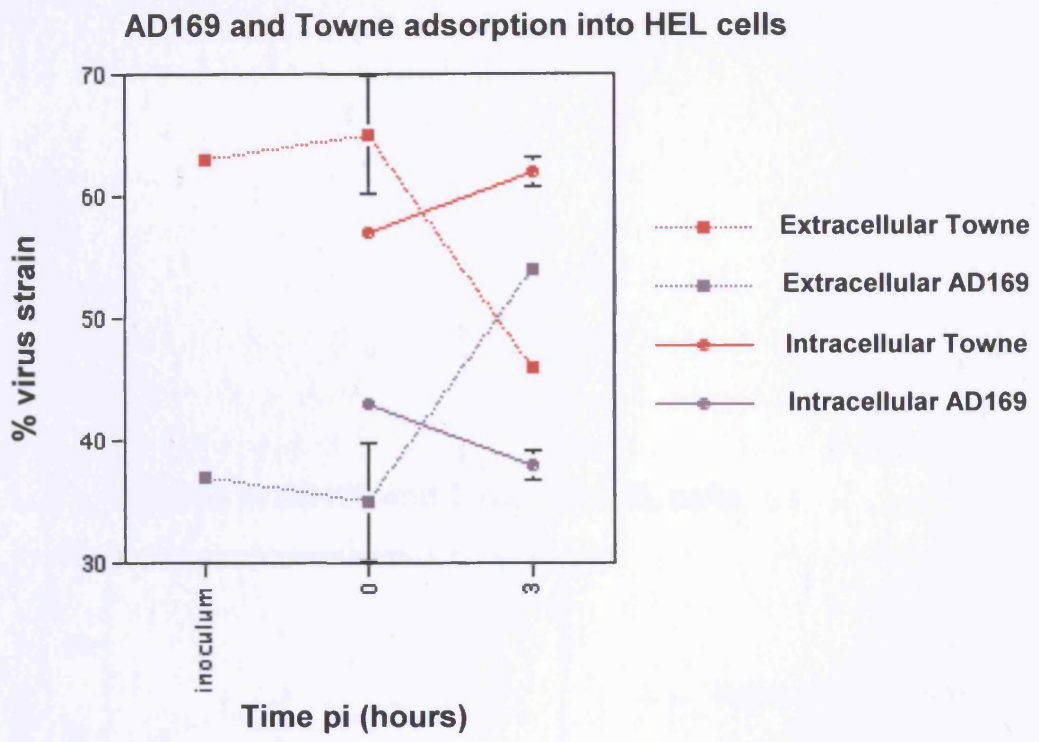


Figure 3.7. (A) Levels of Towne (T) and AD169 (A) after infection of HEL cells with a mixture of these virus strains, consisting of 63% Towne and 27% AD169. **(B)** DNA fragments separated on 3% agarose gel after RFLP.

(A)



(B)

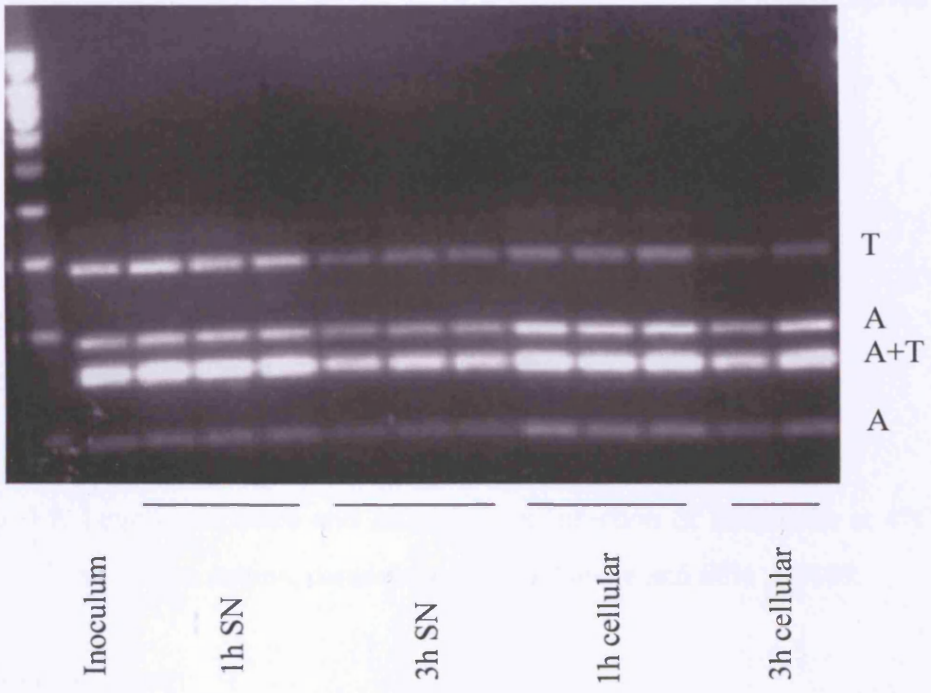


Figure 3.8. (A) Levels of AD169 and Towne after co-infection of HEL cells with equal amounts of the two virus strains. **(B)** DNA fragments separated on 3% agarose gel after RFLP.

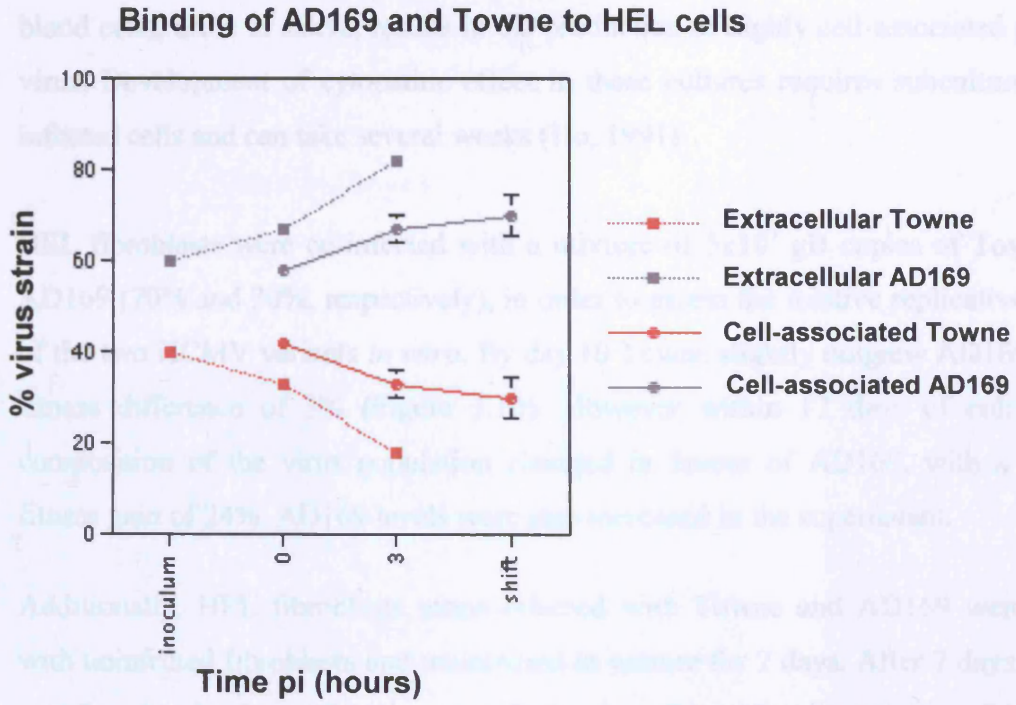


Figure 3.9. Levels of Towne and AD169 after infection of HEL cells at 4°C with a mixture of these virus strains, consisting of 40% Towne and 60% AD169.

*Shift=Shift to 37°C

3.2.4.3 Determination of replicative fitness

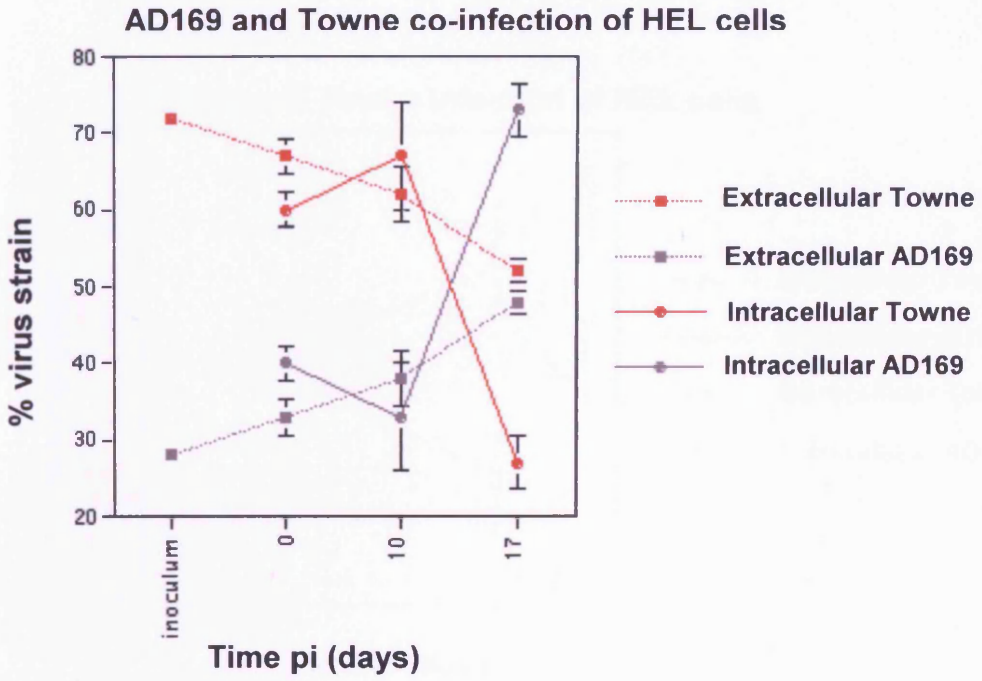
Serial passage and adaptation of HCMV strains in fibroblasts is associated with several phenotypic changes in the passaged strains. The inoculation of fibroblasts with laboratory-adapted HCMV strains leads to the development of cytopathic effect in less than a week, and a single round of replication resulting in cell free virus in the supernatant requires less than 96 hours to complete (Stinski, 1990). In contrast, inoculation of fibroblast cell cultures with HCMV from patient samples, such as white blood cells, urine or saliva, results in the production of highly cell-associated progeny virus. Development of cytopathic effect in these cultures requires subculture of the infected cells and can take several weeks (Ho, 1991).

HEL fibroblasts were co-infected with a mixture of $5x10^7$ gB copies of Towne and AD169 (70% and 30%, respectively), in order to assess the relative replicative fitness of the two HCMV variants *in vitro*. By day 10 Towne slightly outgrew AD169 with a fitness difference of 3% (Figure 3.10). However within 17 days of culture, the composition of the virus population changed in favour of AD169, with a relative fitness gain of 24%. AD169 levels were also increased in the supernatant.

Additionally, HEL fibroblasts mono-infected with Towne and AD169 were mixed with uninfected fibroblasts and maintained in culture for 7 days. After 7 days, Towne was found to be the predominant population in cells, with a fitness gain of 13.5%. It also accounted for 80% of the extracellular virus population (Figure 3.11).

Overall, these results indicate that the fitness diferences between AD169 and Towne is variable and dependent upon the status of the virus inoculum. When using a cell-free virus inoculum, AD169 outgrew Towne within 17 days of culture, with a fitness gain of 24%. When cell-associated virus was used as the inoculum, Towne predominated in both cells and SN, indicating that it is more efficient in mediating cell-to-cell spread of infection and releasing virus into the medium. Cell-to-cell spread may be important *in vivo*, and under such conditions Towne appears to be fitter.

(A)



(B)

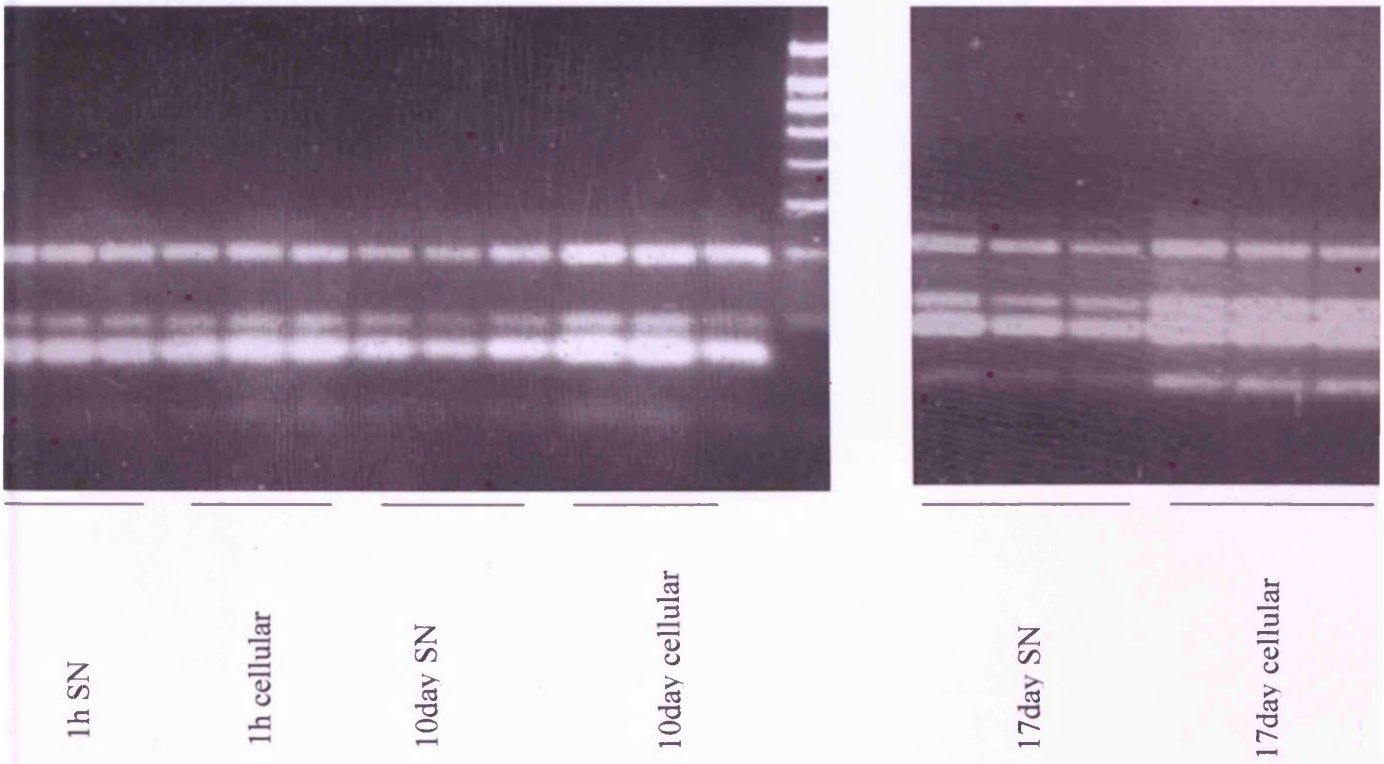
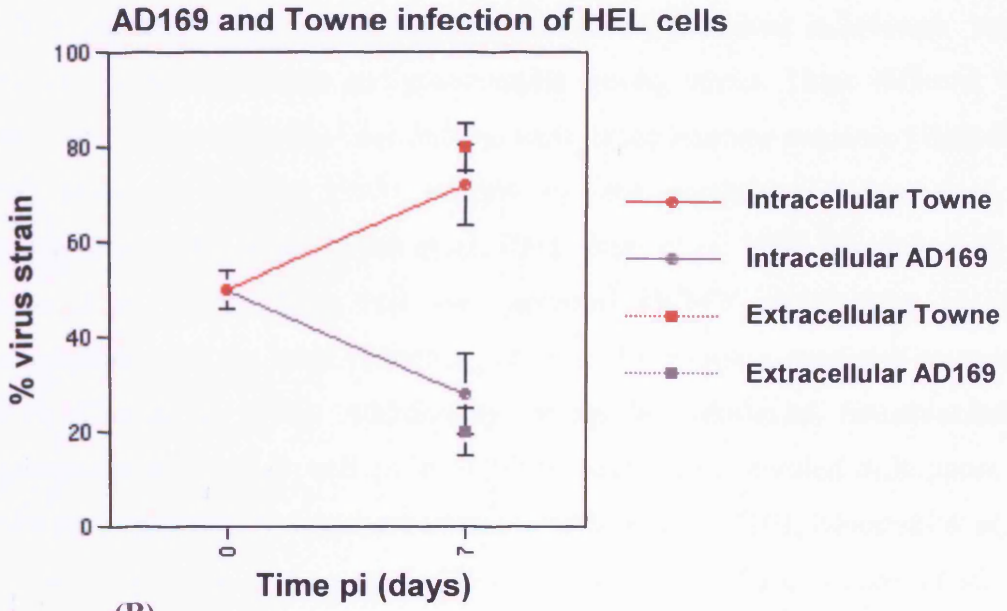


Figure 3.10. **(A)** Levels of AD169 and Towne after co-HEL cells were infected with a cell-free mixture of the two strains at a low MOI. **(B)** DNA fragments separated on 3% agarose gel after RFLP.

(A)



(B)

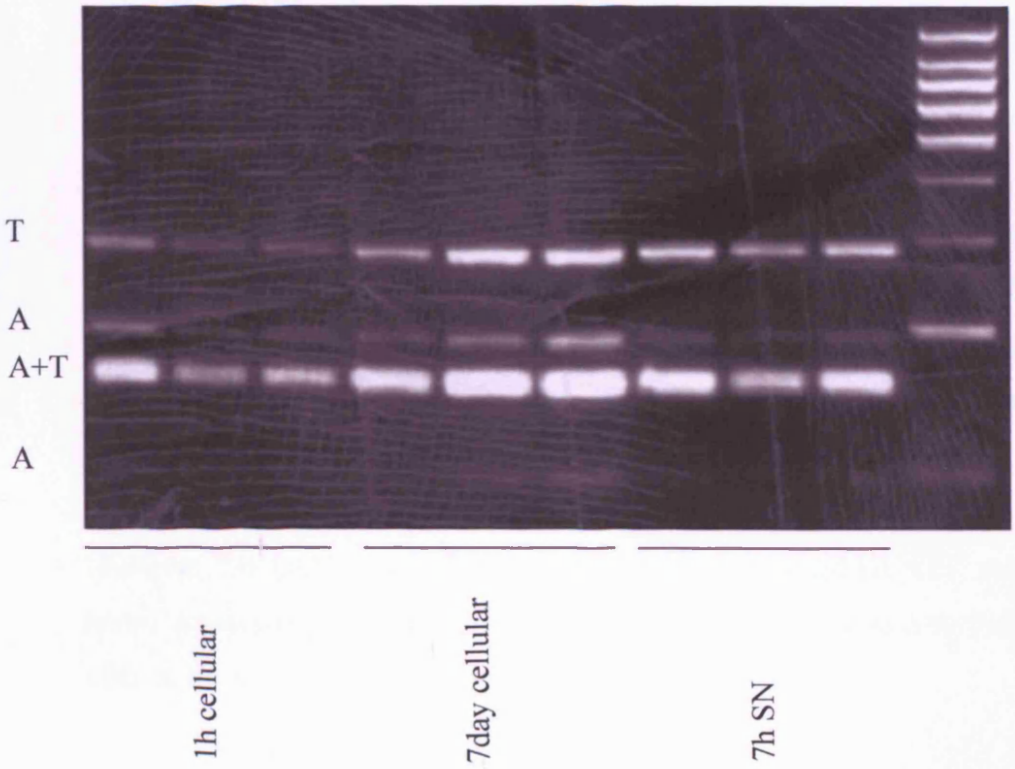


Figure 3.11. **(A)** Levels of AD169 and Towne after co-infection of HEL cells. HEL cells mono-infected with Towne and AD169 (50/50%) were mixed with uninfected fibroblasts and maintained in culture for 7 days. **(B)** DNA fragments separated on 3% agarose gel after RFLP.

3.3 Discussion

HCMV causes a variety of diseases in immunocompromised individuals, including retinitis, mental retardation and pneumonitis among others. These different clinical outcomes may be related to host factors, such as the immune response (Riddell *et al*, 1992, Mach and Landini, 1993), but also to viral properties (Quinnan *et al*, 1984, Plotkin *et al*, 1989, Torok-Storb *et al*, 1993, Fries *et al*, 1994, Sinzger *et al*, 1997, Gerna *et al*, 2000). High and low passaged HCMV strains vary in genome composition, with the latter containing an extra 13kb region, predicted to encode 19 ORFs (Cha *et al*, 1996). Additionally, assays in endothelial, hematopoietic and macrophage cultures, as well as in SCID-hu mice, have revealed differences in the replication capacities among these strains (Waldman *et al*, 1991, Mocarski *et al*, 1993, Minton *et al*, 1994, Brown *et al*, 1995, Plachter *et al*, 1996, Sinzger *et al*, 1997). These genotypic and phenotypic differences are believed to be the result of extensive propagation in fibroblast cell cultures (Sinzger *et al*, 1999).

To evaluate a possible link between genotype and replication rate, fibroblast cultures were infected with HCMV strains AD169 and ToledoE. The efficiency of replication of AD169 exceeded that of ToledoE, by 18-fold, as measured by the number of viral genomes in the infected cells. Calculation of the viral load of each of these strains showed that AD169 replicated better than ToledoE in fibroblasts, reaching $1.4x10^9$ genome equivalents/μg DNA, compared to $8x10^7$ genome equivalents/μg DNA of ToledoE. Furthermore, assessment of the replication of ToledoE in a different cell type, HUVEC, indicated that the virus replicated to higher levels in fibroblasts. However, the doubling times of ToledoE in fibroblasts and HUVEC were 6.9 and 7.3 hours, respectively, suggesting that infected fibroblasts and endothelial cells produce virus at the same rate, but at varying amounts.

The adsorption, internalisation and replication rate (fitness) of HCMV, are processes that allow productive infection to occur. In order to investigate subtle differences in replication capacity, I performed pair-wise experiments in cell culture to compare the ability of HCMV strains AD169 and Towne to bind to fibroblasts. Towne was found to bind to HEL cells with higher affinity compared to AD169. A study by Taylor *et al* has shown that AD169 and Towne bind to the same receptor on fibroblasts, with

similar affinity (Taylor and Cooper, 1989). However, the Towne strain used in those experiments did not contain portion of the UL/b' region. This could account for the contradictory data and suggests a role for the UL/b' region in virus binding.

In the competition experiments, the relative levels of replicative fitness of two HCMV strains, AD169 and Towne, were estimated under controlled *in vitro* conditions, by measuring the rate of change in their proportion as they replicated in cell cultures, based on the restriction endonuclease pattern of the gB gene. Virus cell-to-cell spread and entry of extracellular or cell-free virus particles frequently share mechanistic details, e.g., the use of similar fusion machinery, but cell-to-cell spread can involve intracellular and extracellular events that determine virus delivery to cell junctions and may be more important *in vivo*. Direct cell-to-cell spread of viruses in tissues is a complex, poorly understood process. A number of viruses have become very adept at moving from an infected cell to an adjoining uninfected cell, specifically at sites of cell-cell contact (Johnson and Huber, 2002). Cell-to-cell spread is typically rapid and efficient, partly because viruses and their cell surface receptors are in close proximity, and viruses can move across the narrow spaces between cells, protected from the effects of neutralizing antibodies and other immune system components. When using a cell-free virus inoculum, AD169 outgrew Towne within 17 days of culture, with a fitness gain of 24%. When cell-associated virus was used as the inoculum, Towne predominated in both cells and SN with a fitness gain of 13.5%, indicating that it is more efficient in mediating cell-to-cell spread of infection and releasing virus into the medium. The amount of cell-associated infectious virus was consistently higher than in supernatant culture medium in all the experiments. These results indicate that fitness differences between AD169 and Towne are variable and highly dependent upon the status of the virus inoculum. Since cell-to-cell spread may be more important *in vivo*, under such conditions Towne appears to be fitter. The different properties of Towne and AD169 could be the result of genomic diversity between the two strains and this will be addressed later in this thesis (Chapter 5).

A study by Mousavi-Jazi *et al* comparing the growth phenotypes HCMV isolates *in vitro*, showed that the efficiency of replication of rapidly replicating viruses exceeded that of the slowly replicating viruses by 10-1,000 fold as measured by the virus in culture supernatants (Mousavi-Jazi *et al*, 2000). This difference in replication was not

related to gB genotypes or antiviral susceptibility, but to the expression of early viral proteins. My data indicate that the replication rates of HCMV AD169 and ToledoE strains differed in fibroblasts cell cultures. Additionally, infection of fibroblasts and HUVEC with ToledoE resulted in cell type-specific replication levels. Overall, the results in this chapter illustrate further phenotypic differences between high and low passaged HCMV variants, such as binding onto cells and spread of infection. Deciphering the mechanisms underlying HCMV's interactions with the host will not only contribute to our understanding by which HCMV causes disease, but also provide the foundation for the development of effective approaches for intervention. However, there are limitations to the experimental approaches used here in interpreting the mechanisms and the global impact of these replication differences in cell/viral transcription. Consequently, the subsequent chapters within this thesis have exploited gene array technology to extend these investigations on the replication of different virus strains in the same cell type and the same virus strain in different cell types.

Chapter 4
Construction of a HCMV-human array and expression of the UL/b' region

4.1 The HCMV-human array

4.1.1 Introduction

Viral gene function acts within the context of the host cell. Much of molecular biology is predicated on the fact that the expression pattern (cell type, time, level) of a gene relates to its function, consequently the genes expressed by a cell determine its functions and behaviour. The application of DNA array technology to virology has broadened our knowledge of cellular responses to infection and virus control of host gene expression (Kellam, 2000). Additionally, a single array experiment can reveal the complete gene expression programme of the viral genome (transcriptome). The first virus to have its transcriptome dissected using DNA arrays was HCMV (Chambers *et al*, 1999). This study used a microarray containing oligonucleotide probes for all HCMV ORFs, spotted on glass slides. The array was used to classify all viral genes by kinetic class following infection of fibroblasts with HCMV AD169, with the use of classical metabolic inhibitors. Triplicate experiments were performed in order to assess the significance of expression changes.

High-density DNA arrays of host genes can offer an unparalleled view of the transcriptional events that underlie the host response to viruses. A number of studies have shown that DNA arrays can reveal changes in host gene expression that occur upon virus infection (see section 1.13). Zhu *et al* have reported one of the first analyses of the interaction of a human pathogen with a host cell using arrays, which led to the identification of a number of interesting features of HCMV infection (Zhu *et al*, 1998). Overall, the simultaneous analysis of the expression of thousands of genes can identify the features of the "conversation" between host and pathogen, novel strategies for prophylaxis and therapy and prognostic markers of outcome (Kellam *et al*, 2003).

Because such global analysis of viral and host gene expression has already provided significant clues into the pathogenesis of viral disease, the aims of this chapter were to create and to test DNA arrays for the expression analysis of a subset of HCMV genes, especially these that are unique in clinical strains of virus rather than the lab adapted

strains of HCMV, and approximately 5,000 human genes during productive HCMV infection *in vitro*.

4.1.2 Results

4.1.2.1 Production of the HCMV-human array

An HCMV-human microarray was created to allow the analysis of both virus and host gene expression during HCMV infection of different cell types, namely fibroblasts and endothelial cells. This glass, spotted microarray contains probes for both virus and human genes.

To represent the coding potential of the HCMV UL/b' region genome on the array, a set of PCR primers were designed to amplify around 200-300bp from the 5' end of all the predicted HCMV UL/b' region ORFs, namely UL133 to UL151 (3' end for UL147 and UL148 ORFs), along with gB, UL18, UL130 and UL132. These latter four probes will provide signatures for the known classes of HCMV gene expression, namely gB (early), UL18 (late). The primer pairs were used to amplify probe sequences from genomic viral DNA are listed in Table 4.1 along with the gene name and corresponding region of the HCMV genome. The sequences of the primers used can be found in Table 2.1.

The HCMV array probes were cloned into pGEM-T Easy vector. The identity of each cloned array probe was confirmed by both PCR with virus probe-specific primers (Figure 4.1) and by DNA sequence analysis (see section 2.3.6). All amplified clones were the correct sequence, as determined by BLAST searches of the clone sequences against the non-redundant nucleotide database of GenBank (www.ncbi.nlm.nih.gov/BLAST). This analysis also provided a measure of potential probe cross hybridisation with other host or viral sequences. No array probes were found to have homology to other sequences beside the sequence they represent.

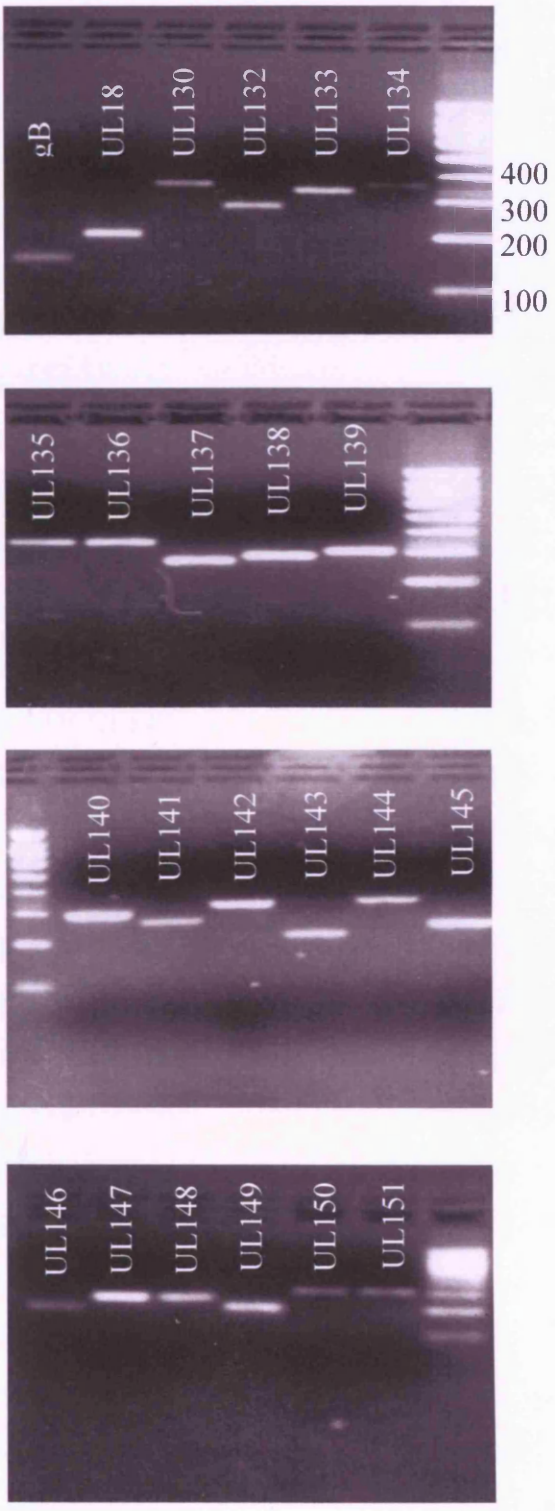


Figure 4.1. PCR of HCMV array clones with clone-specific primers to allow identification of correct clones. Agarose gels, showing products from 23 unique HCMV array clones after amplification with specific primers. All clones are of the correct size. All amplifications were performed using purified plasmid DNA. Size marker in base pairs is included.

ORF / Gene	Sequence[+]
HCMV UL130	13113-13462
HCMV UL132	11961-12236
HCMV UL133	51-380
HCMV UL134	718-1059
HCMV UL135	976-1309
HCMV UL136	2018-2353
HCMV UL137	2631-2890
HCMV UL138	2823-3100
HCMV UL139	3944-4238
HCMV UL140	4492-4780
HCMV UL141	5321-5579
HCMV UL142	6508-6822
HCMV UL143	7370-7580
HCMV UL144	8008-8338
HCMV UL145	8911-9151
HCMV UL146	9579-9768
HCMV UL147	10097-10343
HCMV UL148	11286-11529
HCMV UL149	15758-15950
HCMV UL150	17475-17770
HCMV UL151	17565-17867
Ubiquitin	136-440
TMAP	283-573
PPRT	86-390
GAPDH	61-370
α-tubulin	68-379
MHC class I HLA-B	8-270
β-actin	387-747
HBP	42-340
Ribosomal protein S9	61-370
Luciferase	13-327

TMV	1-319
KSHV K1A	115-420
KSHV ORF4 (CBP)	1142-1448
KSHV ORF6 (ssDBP)	3210-3495
KSHV ORF7	6628-6930
KSHV ORF8 (gpB)	8699-8900
KSHV ORF9 (DNApol)	11383-11680
KSHV ORF10	14519-14810
KSHV ORF11	15790-16002
KSHV ORF2 (DHFR)	18271-18553
KSHV k3 (IE-1B)	19328-19609
KSHV ORF70 (TS)	20801-21104
KSHV K4 (vMIP-II)	21548-21832

* Abbreviations: Tyrosine 3-monooxygenase activation protein (TMAP), pyrophosphate phosphoribosyltransferase (PPRT), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), highly basic protein (HBP).

+ Nucleotide positions relative to the listed GenBank Accession number file

Table 4.1. HCMV UL/b' region array probe clones and their corresponding sequences. The sequences for the probes used as positive and negative controls are also listed.

Common vector primers were designed to amplify the cloned array probes with a minimum of vector sequence (pGEM 5' and 3') (Jenner *et al*, 2001). Previous work has developed PCR conditions to allow the consistent amplification of 5μg of PCR product from pGEM-T Easy clones (see section 2.3.7). Sequences from cloned cellular "housekeeping" genes were also amplified to use as controls using the same conditions. The genes chosen were ubiquitin, tyrosine 3-monooxygenase activation protein (TMAP), pyrophosphate phosphoribosyltransferase (PPRT), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), α-tubulin, MHC class I HLA-B, β-actin, highly basic protein and ribosomal protein S9. Luciferase and tobacco mosaic virus (TMV) 180kD protein genes and KSHV genes were also amplified to use as negative controls. The cloned DNA for the control genes used for amplification was a kind gift from Dr Jenner. PCR amplification (see section 2.3.7) was used to produce approximately 10μg of purified DNA for each of the 46 array elements. The integrity of the PCR reactions was confirmed by agarose gel electrophoresis of 5μl of the purified PCR products and estimating the PCR product size compared to the starting clones, taking into account the increase in size due to the vector specific PCR primers (Figure 4.2).

These probes were added to a 5428-probe set, consisting of probes that were PCR-amplified from sequence verified human cDNA clones (Clark *et al*, 2002). These probes were obtained from the Institute of Cancer Research. The viral and human probe sets were printed onto glass slides to create the 5474-element HCMV-human microarray by Ian Giddings at the Institute of Cancer Research. The arrays used for the analysis of host gene expression in fibroblasts infected with AD169 (used in Chapter 5) contained the same human probe set, but each probe was printed in triplicate. Therefore, these arrays contained 15,000 elements.

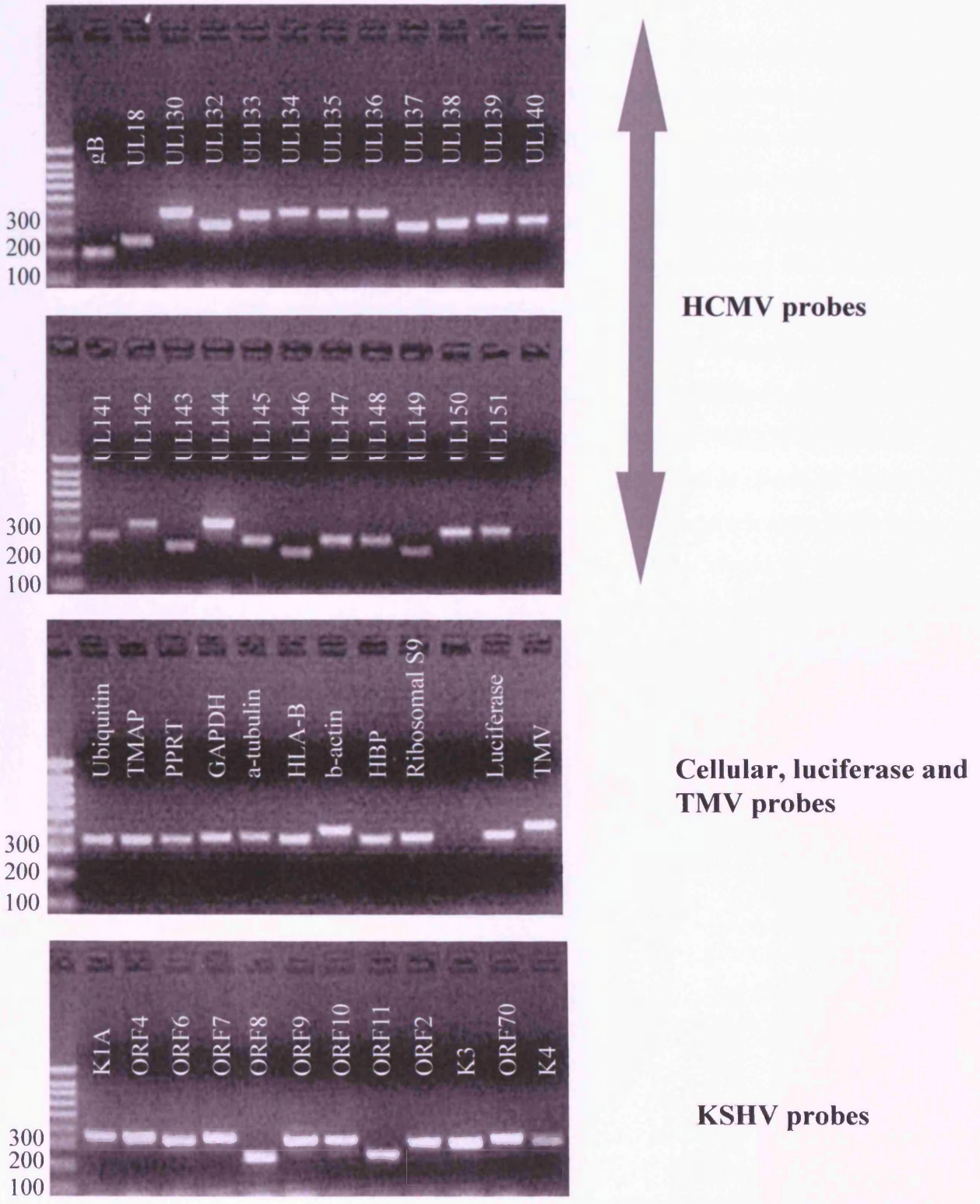


Figure 4.2. Purified array probe DNA. Agarose gels showing purified PCR products for each array probe. In each panel the first lane represents the size markers (Bioline). The lanes containing no bands are water controls.

4.1.2.2 Creation of a common reference RNA sample

The two colour microarrays used in this study make use of a common reference RNA, labelled with cyanine dye, hybridised to all the arrays in the experiment (see section 1.10.2). This common reference RNA allows interarray normalisation and therefore multi-comparative analyses. The reference RNA should ideally provide a signal above background in as many different distinct probes as possible as this maximises the number of expression ratios that can be generated for each sample. The samples analysed in this study are derived from uninfected and HCMV-infected fibroblasts and endothelial cells. Therefore, the reference was designed to reflect this range of cell types and includes RNA purified from endothelial cells infected with HCMV ToledoE and also uninfected peripheral blood mononuclear cells (PBMC) and MRC-5 fibroblasts. The composition of the reference RNA mixture used is shown in Figure 4.3. Batches of each cell type were grown, in order to use the same reference RNA for all the experiments, minimising inconsistencies that may be introduced by the use of different batches of reference RNA. The inclusion of infected cell RNA in the common reference allowed the expression of the virus genes to be detected (Figure 4.3).

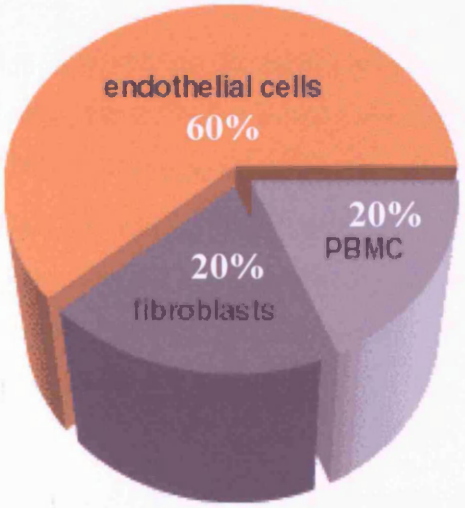


Figure 4.3. Composition of the common reference RNA mixture used with the HCMV-human microarray.

4.1.2.3 Normalisation and filtering of gene expression data

A typical microarray analysis workflow has been documented previously (see section 1.10.2). Before the comparison of array data from multiple samples can take place, the data needs to be filtered to remove unreliable gene expression values and normalised. Normalisation adjusts for differences in the quantity of RNA used and differences in labelling and detection efficiencies for the labels. There are a number of normalisation methods, but one of the most common methods still generally applied to microarrays is global normalisation (see section 1.11.2). Global normalisation is achieved by adding a scaling factor to data from each array so that the median expression ratio in all arrays is equal/adjusted to 1. This makes the assumption that the majority of genes on the array do not change their expression levels when comparing two mRNA samples even if one of the samples is a pooled reference RNA. It also assumes that the expression ratio is independent of signal intensity. This is generally true for genes of medium to high signal intensity but not for low intensity signals. It is therefore necessary to filter out low intensity spots before normalisation. Global normalisation was used to compare expression data derived from the HCMV-human microarrays.

Each host gene was spotted in triplicate on the arrays used in Chapter 6. The median from the triplicate measurements for each gene was calculated and gene expression data were analysed using Cluster. To check the reliability of taking the median value, the standard deviations (SDs) for the triplicate measurements were calculated in Microsoft Excel. This analysis showed that 97.4% of the genes had a standard deviation of less than 1, with the majority of them having a SD=0.2 (Figure 4.4A). Therefore, the reproducibility of the arrays is high.

Gene expression measurements with a low signal to noise ratio can give rise to variation due to imprecisions in detecting low signals. Previous arrays with similar arrays showed that filtering the data to remove elements with a signal to background ratio below 2 in the Cy5 channel and 1.5 in the Cy3 channel was shown to increase the consistency of microarray measurements (Jenner *et al*). Indeed, after calculating the SDs for the same set of gene expression data before and after filtering, the same observation was made (Figure 4.4B).

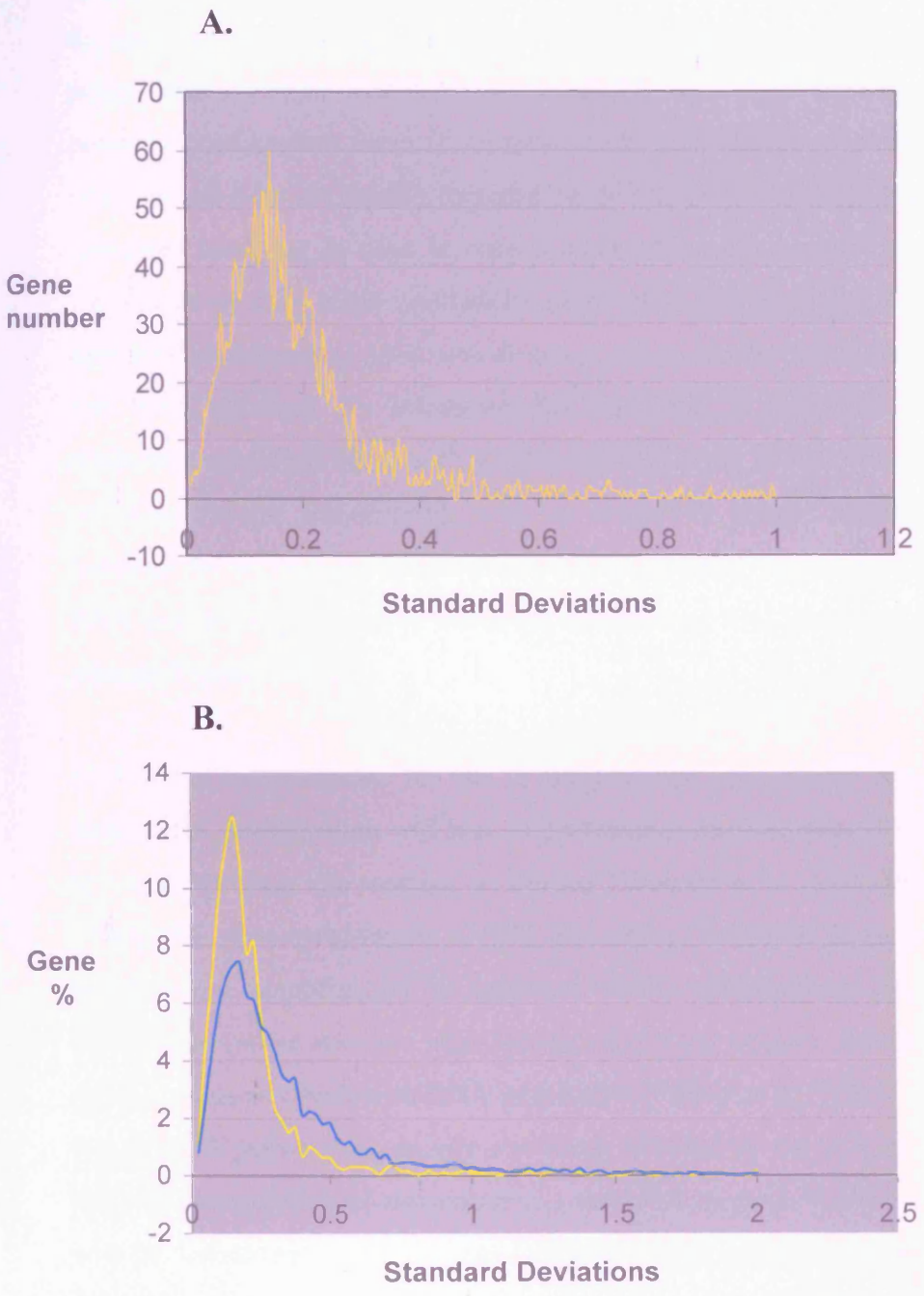


Figure 4.4. (A) Standard deviations (SDs) calculated for the triplicate gene expression measurements on the microarrays (filtered data set) **(B)** Comparison of the standard deviations of gene expression data before (blue line) and after (yellow line) filtering.

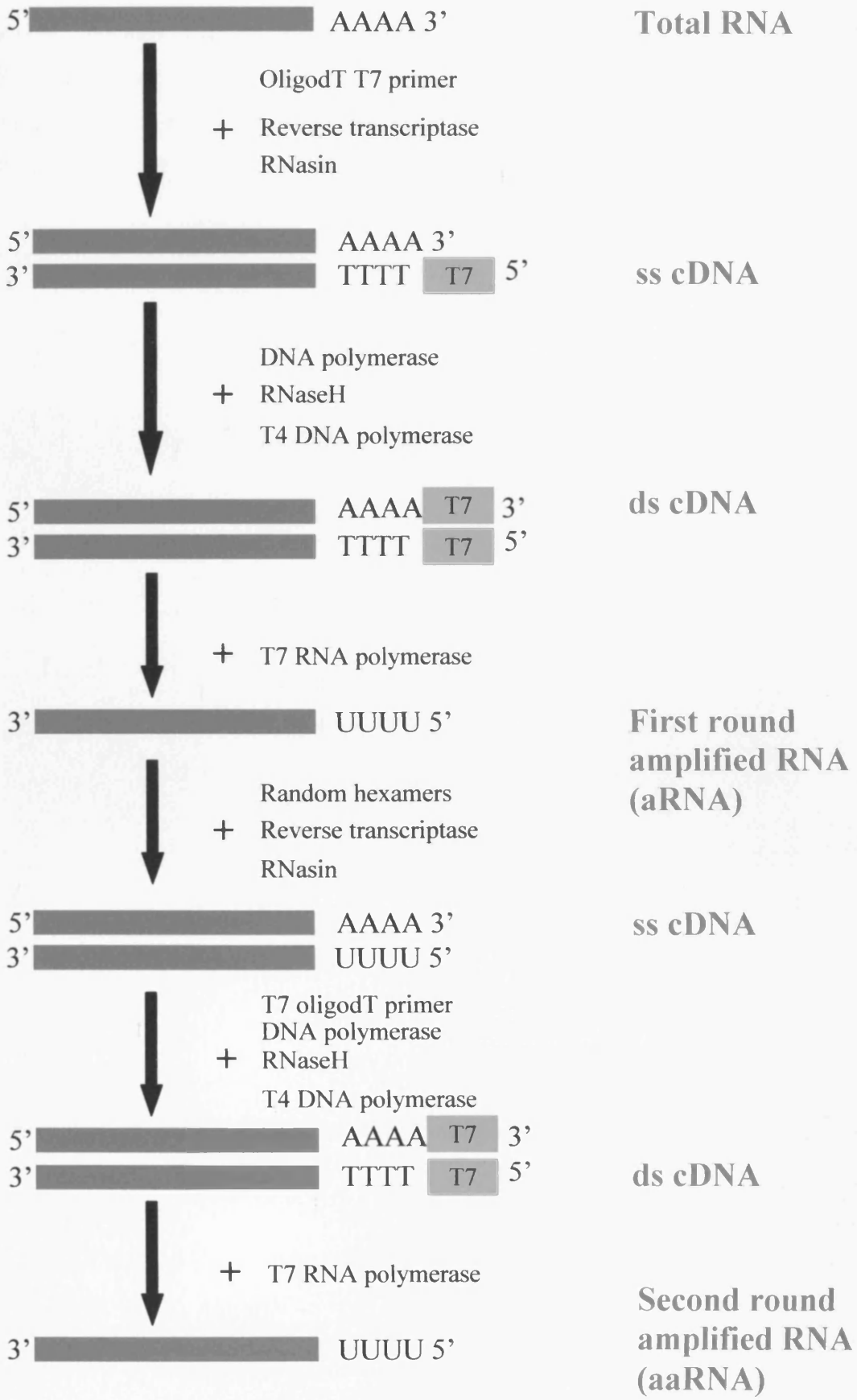
4.1.2.4 Generation of amplified RNA for microarrays

Gene profiling using glass arrays typically requires up to 0.1-1μg of poly(A) RNA to synthesize labelled probe for each hybridisation. However, this poses a limitation for the microarray technology, since it is often impossible to obtain enough RNA from patient blood samples and biopsies. In virology and other "cell exposure" studies, a potential confounding issue in classical molecular biology experiments is produced when not all cells are equally exposed to the treatment. This can lead to multiple cell processes occurring in what is viewed as an otherwise consistent system. One such variable in virology is the multiplicity of infection (MOI). When viral titre is low, the only way to increase an MOI is to decrease cell numbers. This however also results in low RNA recovery. To overcome this limitation, a technique was developed to synthesize amplified RNA (aRNA) (Van Gelder *et al*, 1990). The procedure consists of initial reverse transcription with an oligo(dT) primer bearing a T7 promoter followed by an *in vitro* transcription of the resulting DNA with bacteriophage T7 RNA polymerase, generating hundreds of antisense RNA copies of each mRNA in a sample (Figure 4.5).

PCR is not recommended for use in amplification, since there is a concern that the exponential amplification will lead to preferential amplification of certain cDNAs and to variability from one reaction to another (Wetmur *et al*, 1968, Peccoud and Jacob, 1996). The exponential nature of PCR also makes it difficult to determine the point at which linear amplification is achieved. RNA amplification using the T7 RNA polymerase system does not significantly distort the relative abundance of individual mRNA sequences within an RNA population (Pabon *et al*, 2001). This is most likely due to RNA polymerase activity not being affected by the template sequence or the relative concentration of sequences in a complex mixture. For the few templates that will be transcribed more or less efficiently than others, the aRNA amplification procedure may not generate the same number of aRNA molecules from each template, but their amplification is reproducible from reaction to reaction. Therefore, this allows comparison of expression profiles of different RNA samples following a common amplification protocol.

Amplified RNA obtained from the first round of amplification is the antisense sequence relative to cDNA from which it was transcribed (Figure 4.5). In my experiments, one round of amplification usually resulted in 2-20μg of aRNA. As 5μg of RNA is required for microarray hybridisations, a second round of amplification was performed if the aRNA produced after the first round was less than 5μg (see section 2.7).

Figure 4.5. See opposite page. Outline of the procedure used to synthesise aRNA from total RNA. The synthesis of first strand cDNA is catalysed by the reverse transcriptase in the presence of total RNA and oligodT T7 primer. Second strand cDNA synthesis is performed in the presence of RNaseH and DNA polymerase I. *In vitro* transcription of aRNA is performed by T7 RNA polymerase. To regenerate the T7 promoter sequence for use in the second round of amplification, the first strand cDNA is primed with random hexamers to generate second strand products (see section 2.7).


5' AAAA 3'
Total RNA
OligodT T7 primer
+ Reverse transcriptase
RNasin
5' AAAA 3'
3' TTTT T7 5'
ss cDNA
DNA polymerase
+ RNaseH
T4 DNA polymerase
5' AAAA T7 3'
3' TTTT T7 5'
ds cDNA
+ T7 RNA polymerase
3' UUUU 5'
First round amplified RNA (aRNA)
Random hexamers
+ Reverse transcriptase
RNasin
5' AAAA 3'
3' UUUU 5'
ss cDNA
T7 oligodT primer
DNA polymerase
+ RNaseH
T4 DNA polymerase
5' AAAA T7 3'
3' TTTT T7 5'
ds cDNA
+ T7 RNA polymerase
3' UUUU 5'
Second round amplified RNA (aaRNA)

To formally assess the systematic bias introduced by RNA amplification using my protocol, the expression profile of labelled aRNA-based targets was compared to that of conventional poly(A) RNA-based targets (Figure 4.6). Statistical analysis of expression ratios showed that the microarray sensitivity and precision using aRNA are comparable to the standard hybridisation process using unamplified RNA (r=0.96).

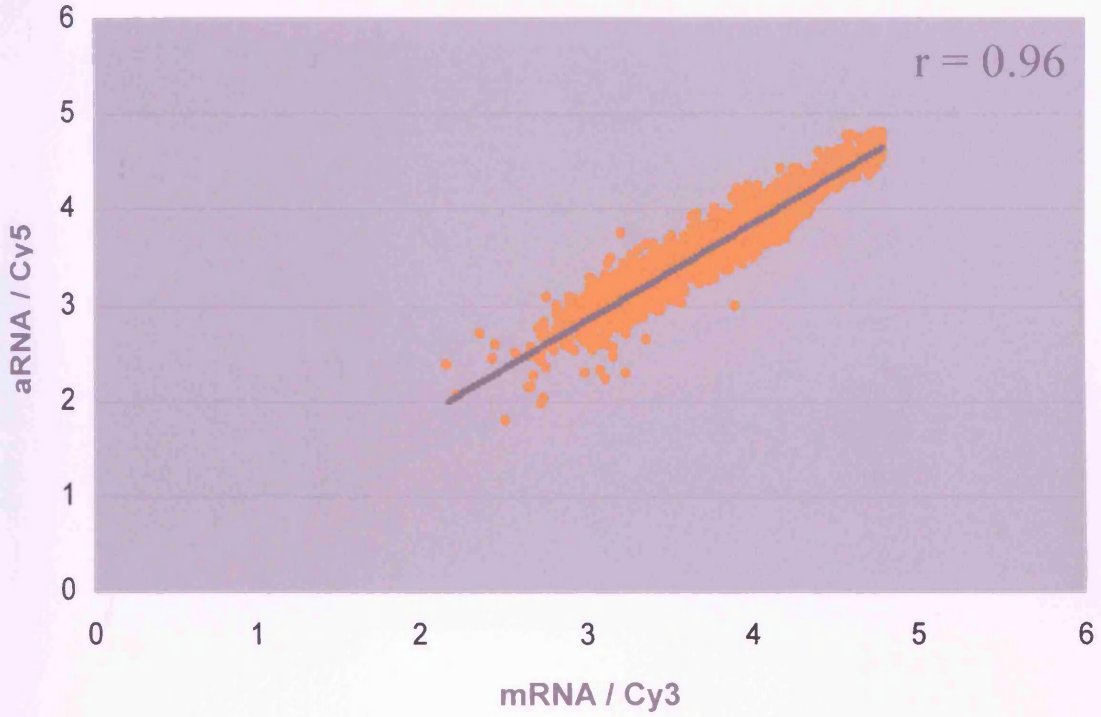


Figure 4.6. Assessment of systematic bias introduced by RNA amplification.

Similarly, I wished to assess the level of bias introduced after a second round of aRNA amplification. I performed pair-wise analysis of normalised microarrays hybridised with aRNA or aaRNA (second round amplified) derived from the same source, relative to a common amplified reference (Figure 4.7). Statistical analysis of the log(2) expression ratios revealed a high correlation coefficient (r=0.83), indicating that the use of second round amplified RNA gives reliable results in microarray hybridisations.

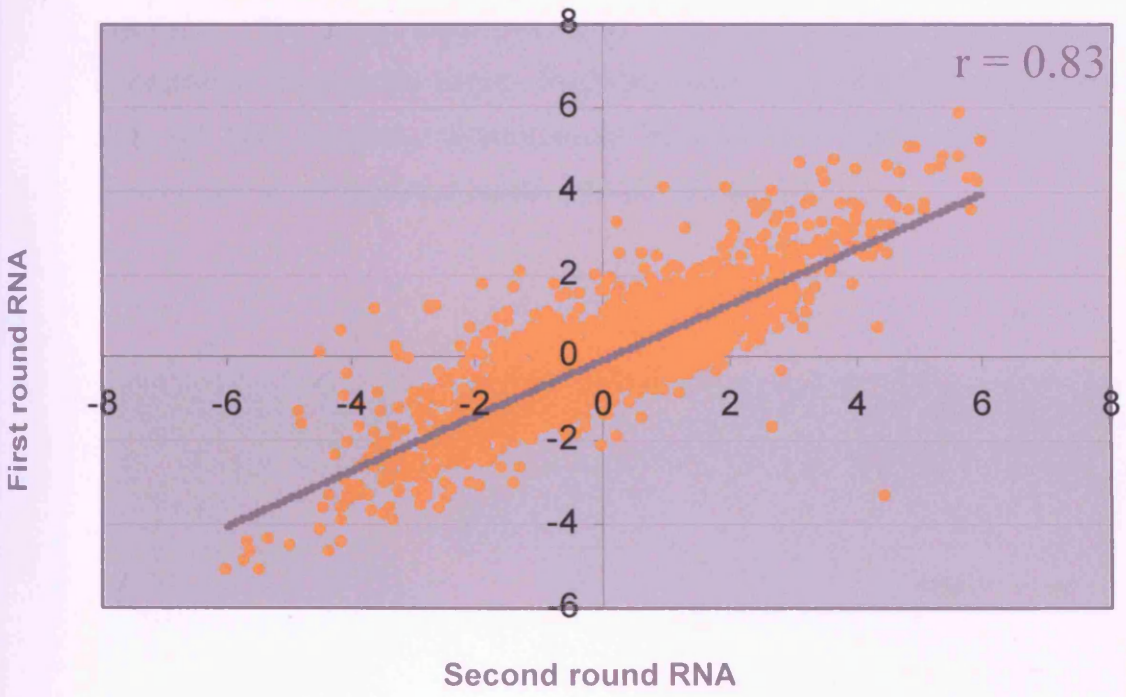


Figure 4.7. Comparison of aRNA and aaRNA targets from endothelial cells infected with human cytomegalovirus (r=0.83).

4.1.2.5 Fluorescent labelling of samples

Microarray analysis is usually carried out by hybridising two fluorescently-labelled probes prepared from cellular RNA, to DNA targets that have been immobilised on a solid support. The most common method for the generation of labelled probes is the direct incorporation of fluorescent nucleotides (ie Cy5 and Cy3) into first-strand cDNA. In my studies, RNA from the sample of interest was always labelled with Cy5, while common reference RNA was labelled with Cy3. To exclude labelling biases introduced by dye variation, aRNA and mRNA targets derived from the same RNA source were labelled with the reciprocal fluorophore, mixed and hybridised to a microarray. The same experiment was then repeated, this time swapping the fluorophores between the targets. Statistical analysis of the log(2) expression ratios from the two microarray hybridisations indicated that use of the Cy5 and Cy3 fluorophores gives consistent results (r=0.86) (Figure 4.8).

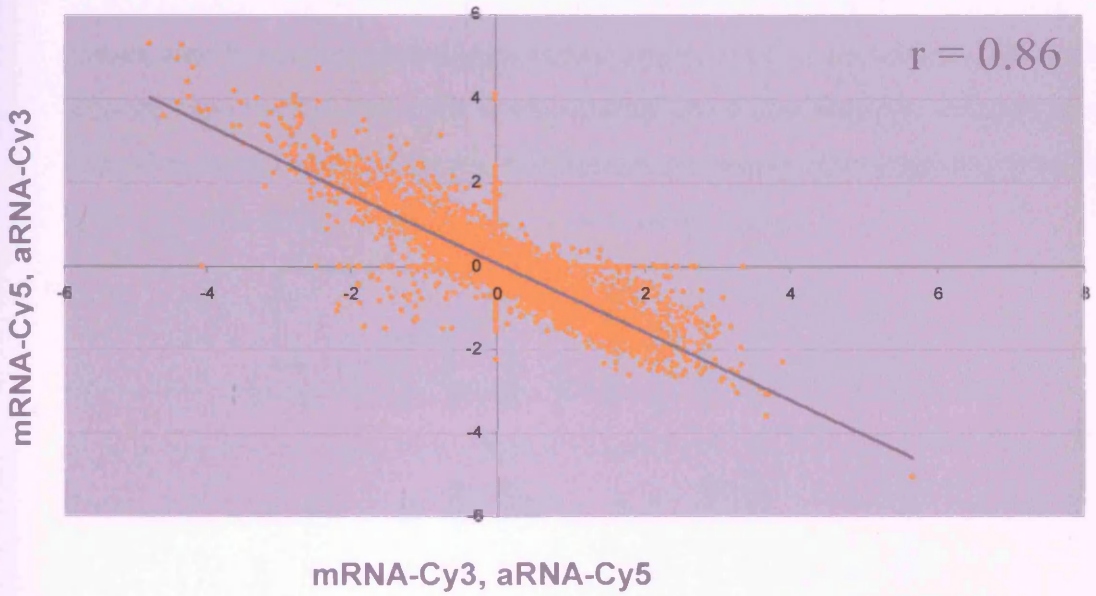


Figure 4.8. Assessement of labelling bias by hybridisation of differentially labelled aRNA and mRNA targets derived from fibroblasts infected with human cytomegalovirus. This is a reciprocal plot, since the ratios for mRNACy3/aRNACy5 are inverted.

4.1.2.6 Cluster analysis of experimental replicates

A requirement for all biological experiments should be repetition to distinguish true reproducibility from experimental variation. DNA microarrays are no exception. In previous sections I used hierarchical clustering to compare arrays. This becomes difficult to interpret when multiple pairwise comparisons can be made between samples/arrays. To aid analysis, the correlation coefficients can be converted to distance measures and pairs of samples/arrays can be joined by a root and two branches based on high correlation which equals low distance. This is known as hierarchical clustering. Here, I use hierarchical clustering to confirm that replicate experiments gave the same gene expression measurements. The log(2) median of ratios from 16 arrays were filtered (see section 5.2.1) and both the arrays and genes clustered using average linkage hierarchical clustering, using the program Cluster (Eisen *et al.*, 1998). The data contain the results of array analysis of 16 different samples. The results show that all replicate samples cluster together because their expression patterns are more correlated with each other than either is to any other sample (Figure 4.9). The cluster dendrogram relates the samples by their expression pattern with branch length being inversely proportional to correlation. The variation between experimental replicates is consistently lower than between different samples. Therefore, I concluded that the HCMV-human microarray gave consistent results.

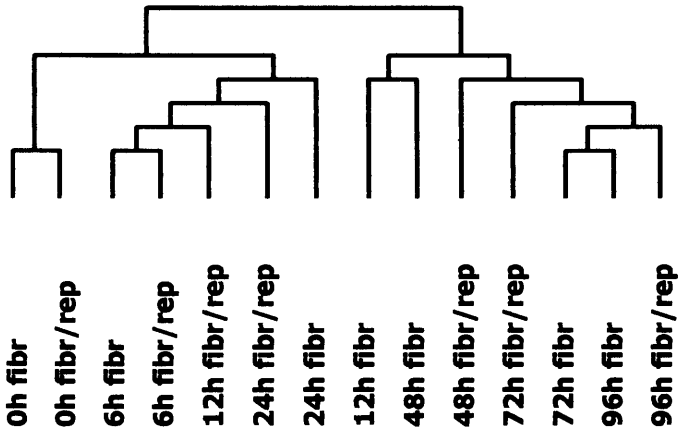


Figure 4.9. Cluster analysis of experimental replicates. Hierarchical clustering of 14 samples (one per array) based on a filtered set of 2,400 genes. The dendogram relates samples by their gene expression pattern, with samples with similar expression patterns clustered together. Branch length is inversely proportional to correlation. Repeats cluster together, connected by short branches.

4.1.2.7 Efficiency of cDNA synthesis during aRNA generation

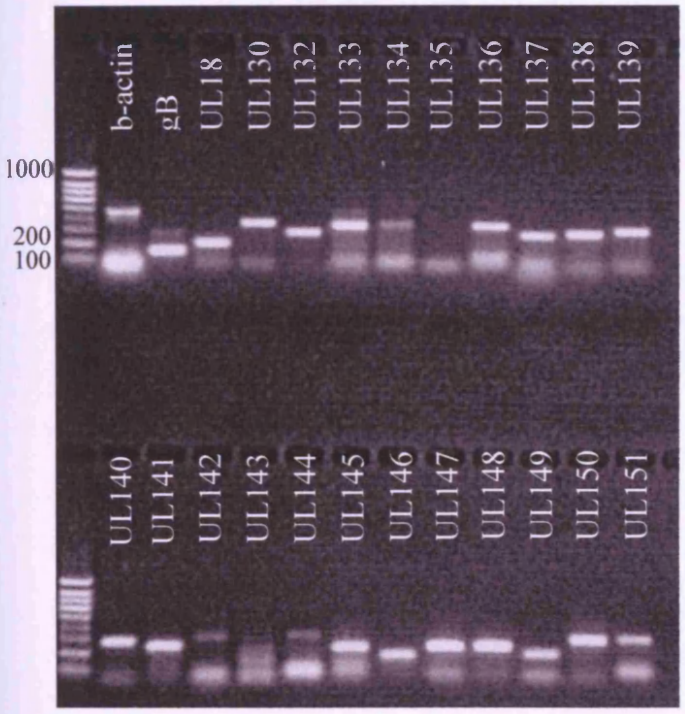
Most eukaryotic mRNAs are polyadenylated; that is, there is a non-template addition of a variable number of A nucleotides to the 3'-ends of eukaryotic mRNA molecules. However, it is not known whether all of the HCMV UL/b' region genes are polyadenylated. The first step in the RNA amplification process involved the generation of first strand cDNA from total RNA, using an oligodT primer. Additionally, priming with anchored oligodT will direct the start of the synthesis of cDNA from the 3' end of the polyA tail. This is desirable if the hybridisation target probes on the microarray are derived from the 3' region of transcripts. However on my microarrays, HCMV probes were derived from the 5' end of genes. This could introduce a problem in hybridisation efficiency if there is incomplete cDNA synthesis. To determine the impact of these factors I tested whether all HCMV UL/b' genes can be detected following oligodT primed cDNA synthesis.

Initially, cDNA was generated from 1μg of total RNA extracted from late-stage HCMV-infected cells, using the oligodT primer. The cDNA was then subjected to PCR analysis using specific primers for the HCMV UL/b' region genes used to generate the array probes (see section 2.7). This resulted in the detection of the majority of the viral transcripts (Figure 4.10A). However UL135 could not be detected, while UL143 was transcribed at very low levels.

To improve the detection of UL135 and UL143, cDNA was generated from 1μg of total RNA extracted from late-stage HCMV-infected cells, using random hexamers. PCR was then performed with specific primers for UL135 and UL143, showing that UL143 was a low abundance transcript, while failing to detect UL135 transcripts (Figure 4.10B). The outcome was consistent with results from studies on the chimpanzee CMV genome, which showed that UL143 is not likely to encode a gene (Davison *et al*, 2003). My results indicate that cDNA synthesis using oligodT is efficient for the majority of transcripts and also indicates that all of the predicted HCMV UL/b' genes produce polyadenylated RNA. Finally, these data confirm the computational analysis of HCMV (Davison *et al*, 2003), showing the absence of a

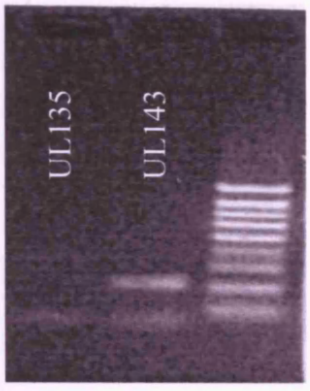
UL143 ORF and adds UL135 to this category of predicted but not transcribed and therefore not functional sequences.

(A)



cDNA primed with oligodT primer

(B)



cDNA primed with random hexamers

Figure 4.10. Agarose gels illustrating the transcription pattern of the HCMV UL/b' genes in late-infected fibroblasts. PCR with specific primers for the viral genes was performed from cDNA primed with **(A)** an oligodT primer and **(B)** random hexamers.

4.2 Expression of the UL/b' region of Toledo

4.2.1 Introduction

In 1990, sequence analysis of the laboratory strain AD169 by Chee *et al* predicted that the virus encoded 208 predicted open reading frames, encoding approximately 189 unique proteins, due to duplication and splicing events (Chee *et al*, 1990). At that time, it was the largest viral genome sequence available. Since then, several other betaherpesviruses have been sequenced, including murine CMV (Rawlinson *et al*, 1996), HHV-7 (Megaw *et al*, 1998) and rat CMV (Vink *et al*, 2000). In 1996 it was reported that 22 open reading frames present in HCMV clinical isolates and the low passaged Toledo strain, termed UL/b' region, were not found in AD169 and other high passage laboratory strains of the virus (Cha *et al*, 1996). However, despite the high level of gene homology between Toledo and wild type virus, some differences exist (Prichard *et al*, 2001). Therefore, no laboratory strain can be considered genetically complete. Since the sequence of the AD169 genome is still being further characterised and there is still no data on HCMV sequences derived from clinical samples, the full wild type genomic composition remains unclear. Recently, in an effort to improve the interpretation of HCMV, its sequence was compared to the chimpanzee CMV sequence (Davison *et al*, 2003), the closest known relative of HCMV. In this study it was proposed that AD169 contains 145 genes, while the wild type HCMV possibly encodes 164 to 167 genes. Because of the complexity of the HCMV genome, function has not yet been assigned to a large number of genes. This is particularly true in the case of the UL/b' genes, which have only recently been discovered. As summarised in the introduction, herpesvirus genes are expressed sequentially in three phases, termed immediate early (IE or α), early (E or β) and late (L or γ), according to the onset of transcription (Stinski, 1990). The timing of a gene's expression also gives clues as to its function (see section 1.3).

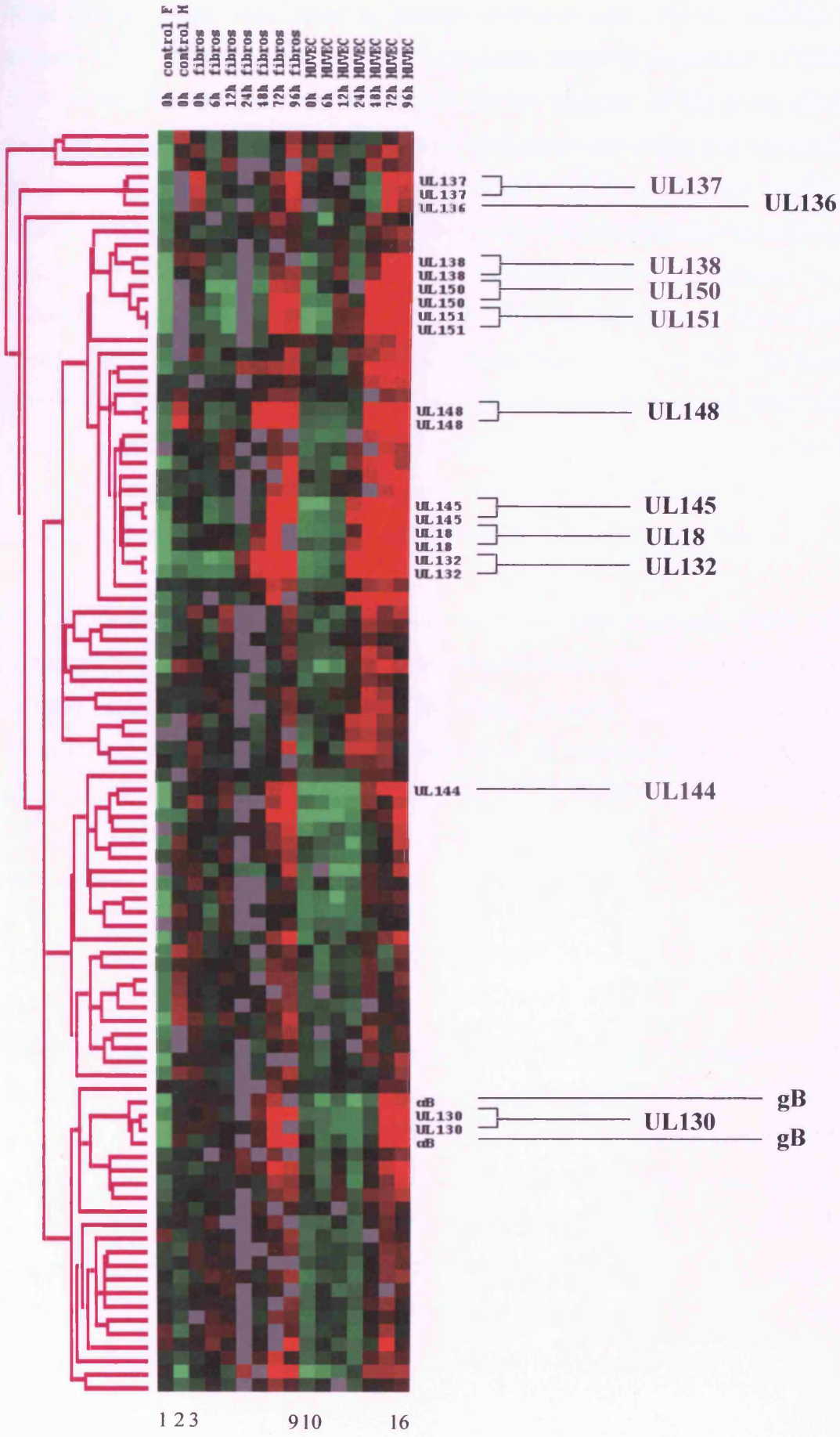
DNA array technology is a powerful tool for identifying programmes of herpesvirus gene expression, since it can provide simultaneous measurements of gene expression. In 1999 the generation of a viral DNA array chip for parallel expression measurements of nearly all known open reading frames of HCMV was described (Chambers *et al*, 1999). RNA extracted from fibroblasts infected with Towne was

hybridised to chips containing DNA probes for all the genes present in AD169 and four from Towne (UL147, UL152-UL154). Viral genes were then categorised as immediate-early, early or late according to their expression in the presence of cyclohexamide and inhibitors of viral replication (ie ganciclovir). The CMV chip did not detect all the IE genes, however no false positive signals were detected. Additionally, the majority of the genes were found to belong to late expression classes, with the UL/b' UL147 gene being classified as an E-L gene. Overall the study described the expression of 151 ORFs, showing 90% agreement with published studies. The expression pattern of the majority of the HCMV UL/b' genes and how this relates to the ordered series of events that occurs during replication, remain largely unknown. The aims of this section of the chapter are to use the HCMV-human array to analyse HCMV UL/b' gene expression during lytic replication in both fibroblasts and endothelial cells.

4.2.2 Gene expression analysis by HCMV-human arrays

The HCMV-human array was used to monitor virus gene expression in fibroblasts and endothelial cells after infection with ToledoE. Cells were harvested over a 96-hour time-course, total RNA was purified, amplified and labelled with Cy5 and hybridised to the arrays. The reproducibility of the arrays was confirmed by performing duplicate hybridisations representing 0 (time of virus inoculum removal), 6, 12, 24, 48, 72 and 96 hours after infection (see section 4.1.2.6), showing that duplicate samples give highly similar gene expression patterns. The data from the time-course were ordered with a self-organising map and the genes grouped by hierarchical clustering. Hierarchical clustering separated the genes into branches in which genes share similar patterns of expression. Analysis showed arrays were specific with no HCMV signals detected in the absence of HCMV infection and no cross hybridisation to KSHV gene probes. This analysis showed the viral genes were all grouped in the same cluster (Figure 4.9). To aid interpretation of the gene expression values, each time point was expressed as a ratio change relative to time 0, allowing the effective increase in each gene expression over time to be assessed. In certain cases (ie early time points) viral transcripts were not detected, probably because they were below the threshold of sensitivity of the microarrays.

Figure 4.11. Hierarchical clustering of gene expression data. This figure is an expanded view of the cluster containing the viral genes. Each row represents a separate gene on the array; each column represents the results from one array. Shades of red indicate detectable expression above and shades of green illustrate expression below the median level of expression for each gene (black). The dendrogram on the left represents the gene expression distance of the patterns of expression between different genes (short branch lengths equate to similar gene expression profiles). The gene names are listed on the right hand side. Lanes 1 and 2 represent uninfected fibroblasts and endothelial cells, respectively. Lanes 3 to 9 show time points after infection of fibroblasts with ToledoE, while lanes 10 to 16 show the array results from ToledoE-infected endothelial cells.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Viral gene expression was found to increase over time after infection with the virus (Figure 4.11). Different genes reached significant levels of expression at different time points. By 48 hours, intense signals for the majority of the genes could be detected, suggesting a prompt escalation of viral gene expression. The expression of gB increased significantly (~4 log(2)) within 48 and 72 hours after infection of fibroblasts and HUVEC, respectively, consistent with its classification as an early-late gene. UL18 transcripts were also detected at late time points after infection. UL18 is homologous to MHC class I and although there is contradictory data about its function, it is thought to have a role NK evasion (Reyburn *et al*, 1997). It has been shown that cells infected with HCMV exhibit a marked decrease of MHC class I molecules on their surface, which makes them susceptible to NK cell lysis. Therefore, the expression of UL18 at late time points is concomitant with the reported down-regulation of MHC class I molecules and consistent with its proposed function.

UL130 levels increased by ~4 log(2) at 72 hours after infection, while UL132 transcription occurred at 96 hours. 8 of the 19 genes that are missing from laboratory-adapted strains were detected using the arrays, namely UL136-UL138, UL144, UL144, UL148, UL150 and UL151. These were all transcribed at late time points after infection. The highest level of expression was observed for UL150, whose transcript levels increased 32 times, by 72 and 96 hours in fibroblasts and HUVEC, respectively.

4.2.3 Gene expression analysis by RT-PCR

Because array sensitivity sometimes prevents detection of low abundance transcripts, and in order to confirm the array-based gene expresion profiles, I analysed expression of the UL/b' genes by RT-PCR using the array probe PCR primer sets. Total RNA was prepared from several time-points (0, 6, 12, 24, 48, 72, 96h) after infection of fibroblasts and HUVEC with HCMV ToledoE. cDNA was synthesized from 1μg of total RNA primed with the T7 oligodT primer using Superscript II. 1μl of cDNA was then added to a HotStart Taq (Qiagen) PCR mix, containing specific primers (100ng) for each viral gene. The PCR products were separated on agarose gels.

UL141 and UL148 were expressed within 1 hour of HCMV infection, consistent with immediate early gene functions. UL134, UL142, UL144 and UL146 transcripts started accumulating at 48 hours after infection. Genes encoding proteins that form part of the virus particle are expressed late in the virus life cycle. The rest of the UL/b' genes were detected between 6-24 hours post infection, implying a role in DNA replication, repair and immune evasion. These results show that the HCMV transcriptional profile changes as the virus progresses through the life cycle, with individual genes having distinct expression kinetics. The majority of the genes (UL137, UL138, UL141, UL144, UL145, and UL144-UL150) exhibited the same transcription patterns in both cell types. There was no detectable expression of UL135 and UL143 during infection, consistent with Davison *et al*, while UL142 was expressed only in HUVEC. The remaining genes (UL133, UL134, UL136, UL139, UL140, UL151) were transcribed with delayed kinetics in fibroblasts compared to HUVEC (Mann Whitney, $p<0.05$).

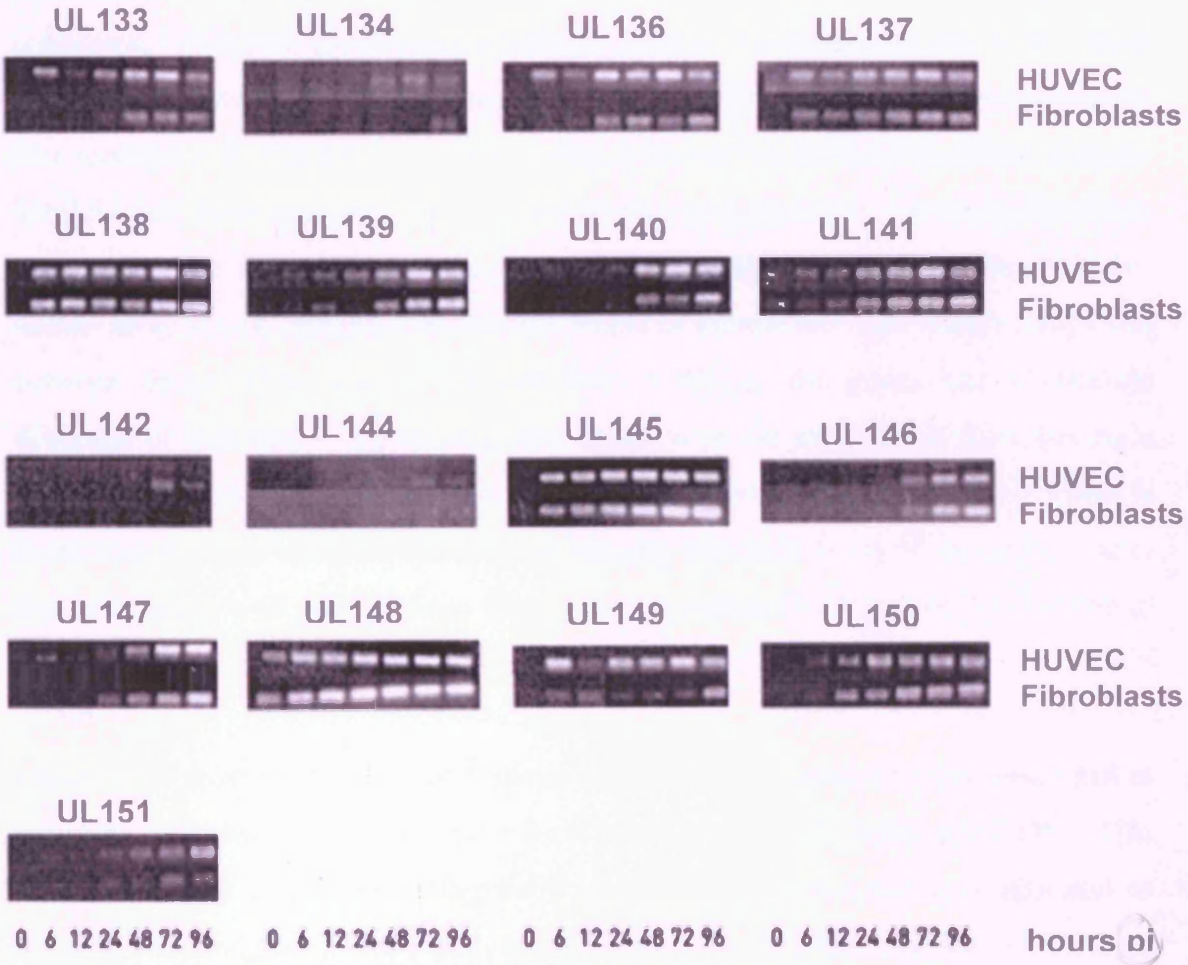


Figure 4.12. RT-PCR with specific primers for the genes located at the UL/b' region of the HCMV genome.

4.3 Discussion

4.3.1 The HCMV-human array

I have created a DNA array to identify the transcription profile of a region of the HCMV genome thought to be crucial for disease pathogenesis *in vivo*. This will enhance our understanding of HCMV behaviour. The array also contains probes for over 5,000 human genes, allowing simultaneous measurements of gene expression for both host and virus. The results from this type of study can unravel interactions between the virus and host and give an insight into disease pathogenesis.

When using arrays for the measurement of gene expression, multiple control experiments are required to ensure the arrays are reliable (Schena *et al*, 1995, 1996, Lockhart *et al*, 1996). The precise identity of the probes on an array has to be confirmed. Firstly, the identity of each cloned array probe spotted on the HCMV array was confirmed by PCR with probe-specific primers (Figure 4.1) and DNA sequencing (see section 2.3.6). Cloned array probes were also checked for the possibility of cross-hybridisation, both against each other and against host genes, by searching GenBank using pair-wise sequence analysis (www.ncbi.nlm.nih.gov/BLAST). The HCMV-human array shows reproducible measurements of expression both within arrays and between arrays. This analysis showed that 97.4% of the genes had a standard deviation of less than 1 among triplicate spots, with the majority of them having a SD=0.2 (Figure 4.4A). Additionally, the variation between experimental replicates is lower than between different samples, confirming the consistency of the array. These results compare well with findings from previous studies (DeRisi *et al*, 1997, Chen *et al*, 1998, Mayne *et al*, 2001).

Many of the most productive applications of functional genomics to date have been in the study of homogeneous cultured cells (DeRisi *et al*, 1997, Heller *et al*, 1997, Chu *et al*, 1998, Iyer *et al*, 1999, Hughes *et al*, 2000). To detect rare messages and to simplify the analysis of complex expression patterns, isolation of the cell type of interest is often essential. Dissection and cell sorting can be used to reduce the complexity of cell types from which RNA is isolated (Sirivatanauksorn *et al*, 2000).

With these techniques, however, it is often difficult to obtain the quantities of RNA required for a transcript profiling technology such as DNA arrays. A technique capable of amplifying small amounts of RNA without significantly distorting the information content of the sample has been described (Van Gelder *et al*, 1990). This technique amplifies the mRNA population by *in vitro* transcription of cDNA. The amplification based on the protocol first described by Eberwine *et al* faithfully maintains relative mRNA levels when starting with 1μg of total RNA (Salunga *et al*, 1999). Application of additional rounds of amplification from much smaller amounts of RNA gives reproducible results for a single RNA sample and allows detection of differences between samples consistent with those detected without amplification (Luo *et al*, 1999, Wang *et al*, 2000). Recently, an optimised protocol has allowed faithful amplification of just 2ng of total RNA (Baugh *et al*, 2001). RNA amplification has been used by several research groups, to generate significant amounts of RNA from biological tissue (Phillips and Eberwine, 1996, Pabon *et al*, 2001, Hu *et al*, 2002). In my experiments, *in vitro* transcription of 1μg of total RNA resulted in the generation of 5-20μg of aRNA, which was sufficient for the microarray hybridisations. Statistical analysis of expression ratios showed that the microarray sensitivity and precision using aRNA are comparable to the standard hybridisation process using unamplified RNA ($r=0.96$). Similarly, the use of second round amplified RNA gives reliable results in microarray hybridisations ($r=0.83$).

The experiments discussed in the first part of this chapter offer controls for the biological interpretation of the array-based expression measurements that take place in the second part of the chapter and in chapters 5 and 6. The HCMV-human array was used to examine (i) HCMV gene expression during active replication of HCMV in fibroblasts and endothelial cells, (ii) host-specific responses to HCMV Toledo and (iii) host responses to different strains of the virus.

4.3.2 Expression of the UL/b' region of ToledoE

The expression of the majority of the HCMV UL/b' genes has not been analysed before. The HCMV-human array and RT-PCR were used to analyse expression of these genes during active replication in fibroblasts and endothelial cells. These technologies provide simple methods of assessing when the expression of viral genes becomes detectable. In this case both methods were required due to inconsistencies in detected low abundance transcripts by microarrays.

Analysis of the array results showed that the viral genes are expressed at different times after infection and with cell type-specific patterns. It is widely accepted that the relative time at which the expression of a gene is detected correlates with the stage of the life cycle in which it is thought to act. UL141 and UL148 were expressed within 1 hour of HCMV infection, consistent with immediate early gene functions. Recent unpublished data presented showed that the expression of UL141 in fibroblasts takes place two days after infection and a function in NK cell evasion was proposed for this gene (Wilkinson *et al*, unpublished data).

UL134, UL142, UL144 and UL146 transcripts started accumulating at 48 hours after infection. The expression patterns observed for UL144 does not agree with previously published data. Lurain *et al* showed that UL144 is a TNFR homologue, expressed early after infection and retained in an intracellular compartment (Lurain *et al*, 1999). In my study UL142 was classified as a late gene. This gene has a role in NK evasion, and combined with previous data on UL18 (see section 4.2.2), shows that both of these genes that are thought to mediate inhibition of NK cell lysis have similar expression patterns. Expression of UL142 was only detected in endothelial cells. Experiments performed by a different group also found it to be transcribed with late kinetics, and also failed to detect expression in fibroblasts (Wills *et al*, unpublished data).

The majority of the UL/b' genes were detected between 6-24 hours post infection, implying a role in DNA replication, repair and immune evasion. This is consistent with the function that has been proposed for UL147 in virus modulation of the

immune system, due to its homology to an alpha chemokine (Penfold *et al*, 1999). UL135 and UL143 transcripts could not be detected in any of the two cell types infected with the virus. This agrees with the study by Davison et al, which discounted UL143 from being a gene (Davison *et al*, 2003).

Some of the HCMV UL/b' region genes share sequence homology with human genes (Table 4.2). Therefore, these genes may represent acquisitions from the host genome. My analysis shows that genes belonging to the same functional group have similar expression profiles and has helped to assign functions to genes for which there is no available information. The classification of the three basic groups of transcripts is generally based however on measurement of transcript abundance in infections where virus-induced DNA and protein synthesis are blocked with metabolic inhibitors. Although it would be useful to monitor expression with inhibitors of translation and herpesvirus DNA polymerase, these disturb the system and, in the case of the inhibition of translation, have wide-ranging effects on the host cell.

HCMV	Homology	Kinetic class	Proposed function
UL137	Protease	-	-
UL139	Neurotoxin	-	-
UL142	MHC class I	Late	NK evasion
UL143	dUTP nucleotide hydrolase	-	-
UL144	TNF receptor	Immediate early	Immune evasion
UL146	α chemokine	-	Neutrophil attraction
UL147	α chemokine	Late	Neutrophil attraction

Table 4.2. Table showing information on HCMV UL/b' genes (homology to cellular genes, expression pattern and current proposed function).

The additional region at the right end of UL in Toledo was described to contain 19 genes absent from AD169 (Cha *et al*, 1996). A recent study, however, has discounted five of these genes, namely UL134, UL137, UL143, UL149 and UL151. The use of DNA arrays and RT-PCR has allowed to test these findings. UL143 transcripts could not be detected in either fibroblasts or endothelial cells, while expression of the rest of the genes was observed, demonstrating these regions of the genome are transcribed. It is clear that the virus expression programme is determined by genome content and

cell type. My data suggest that viral genes are differentially regulated at the level of transcription and may have distinct functions in the virus life cycle.

Chapter 5
Differential modulation of cellular transcriptomes by HCMV Toledo

5.1 Introduction

HCMV genes and induced cellular factors have the potential to exert profound effects on host cell gene expression. The very first steps of HCMV infection, binding and entry, generate a number of cellular signalling responses and the induction of interferon response genes (Albrecht *et al*, 1990, Boldogh *et al*, 1990, 1991, Zhu *et al*, 1997, Navarro *et al*, 1998, Zhu *et al*, 1998, Yurochko and Huang, 1999). This indicates that viral cell surface binding plays a predominant role in stimulating host cell gene expression. Binding of ultraviolet light-inactivated virions to cells can also trigger intracellular signalling cascades (Boldogh *et al*, 1993, Yurochko *et al*, 1995). Additionally, soluble forms of viral proteins gB or gH able to stimulate cells on their own (Yurochko *et al*, 1995, Boyle *et al*, 1999, Simmen *et al*, 2001). It is possible that many of the early changes in cellular genes are not specific to gB or gH, but are caused by the triggering of an innate defensive pathway that detects attachment or entry of a variety of viruses in general and leading to general immune system gene transcription. Alternatively, cell surface binding induced gene expression changes may be necessary for effective viral infection and replication. As infection proceeds, expression of viral proteins then regulates cellular signalling pathways and gene expression to aid viral replication (Stinski, 1977, Albrecht *et al*, 1984, Santomenna and Colberg-Poley, 1990, Rodems and Spector, 1998).

HCMV is an intracellular parasite and therefore relies on host cell to provide functions to allow viral replication, such as DNA metabolism enzymes, DNA polymerase (Hirai and Watanabe, 1976), transcription factors (Boldogh *et al*, 1990, Sambucetti *et al*, 1989, Yurochko *et al*, 1997) and cell cycle genes (Jault *et al*, 1995, Dittmer and Mocarski 1997, Salvant *et al*, 1998, Sinclair *et al*, 2000). The fact that HCMV infection stimulates host cell proliferation suggests a possible involvement in chronic proliferative disorders, such as vascular diseases (Spector and Spector, 1984, Thompson *et al*, 1993, Epstein *et al*, 1996).

Detection of the cellular transcription pathways that are activated or repressed during viral infection can lead to a better understanding of the virus-host interaction and to the discovery of new targets for therapy. DNA array technology has the potential to offer an unparallel view of the transcription changes that underlie the host response to

pathogens. Zhu *et al.* (Zhu *et al*, 1998) reported the first analyses of the interaction of a HCMV with a host cell using microarrays. They catalogued 258 genes whose expression levels varied 4-fold early in the virus replication cycle, up to 24 hours post infection. Some of these were further confirmed by northern blotting. This study and the majority of other studies investigating the replication of HCMV have used human fibroblasts. Nevertheless, HCMV infects a wide variety of cell types *in vivo*, including fibroblasts, endothelial cells, epithelial cells and macrophages. As these cell types are diverse and express different gene sets, the interaction of the virus with the cellular machinery however, may differ from cell type to cell type. This interaction may profoundly influence HCMV replication and pathogenesis. Endothelial cells have an essential role in HCMV pathobiology, since they can function as reservoirs of persistent virus as well as carriers of the virus in the HCMV dissemination pathway (Fish *et al*, 1995). They also are an important component of the inflammatory response, since they are located between blood and tissue and are constantly exposed to circulating leukocytes (Pober, 1999). Finally, they can be induced by cytokines to function as antigen presenting cells (Pober *et al*, 2001).

The combination of DNA arrays and RT-PCR has allowed us to identify the transcription profile of a region of the HCMV genome thought to be crucial for disease pathogenesis *in vivo*, and provide insights into the functions of previously uncharacterised HCMV genes (see sections 4.2.2 and 4.2.3). The aims of this chapter are to use the HCMV-human microarray to identify fibroblast and endothelial cell responses to HCMV infection.

5.2 Results

5.2.1 Sample preparation

To test the hypothesis that HCMV causes specific cell type gene expression changes that relate to disease pathology, gene expression of ToledoE-infected cells was analysed at several time points after infection (0, 6, 12, 24, 48, 72 and 96 hours). Two types of cells were used, MRC-5 fibroblasts and human umbilical vein endothelial cells (HUVEC). Uninfected MRC-5 cells and HUVEC were also included in the analysis. In addition, fibroblasts infected with AD169 or ToledoF were analysed (96

hours). Total RNA was purified from all samples and the quality assessed by agarose gel electrophoresis (Figure 5.1). mRNA was purified, amplified and labelled with Cy5 using RT-PCR. Labelled cDNA was mixed with Cy3-labelled reference RNA and hybridised to the HCMV-human microarray. A common reference RNA mixture was used to permit comparison across the whole sample set (see section 4.1.2.2).

5.2.2 Gene expression in fibroblasts

The arrays were used to determine the host gene response to HCMV Toledo infection of fibroblasts. To confirm the reproducibility of the arrays, duplicate hybridisations were performed for the time-course of infection in fibroblasts, giving consistent results (Figure 4.9). One array was selected for each of the 9 samples and the data were filtered to produce a set of 1,203 human genes. The data were examined by cluster analysis to identify patterns in the gene expression data and identify relationships between the samples. Both the genes and samples were ordered using a self-organising map algorithm, which minimises the differences between adjacent nodes. This ordering was then used to control the orientation of the nodes of the dendrograms subsequently generated by hierarchical clustering. HCMV causes different gene expression patterns at different time points during lytic infection. This resulted in two clusters of gene expression, revealing a demarcation between early and late events in viral replication at the level of the host genes (24-48h) (Figure 5.2). Early-infected cells cluster together, indicating that they share the same gene expression pattern, while all late stage-infected cells form a separate cluster. The two final major branches are composed of cell infected with AD169 and ToledoF, respectively. Their clustering indicates they have similar gene expression patterns compared to cells infected with ToledoE.

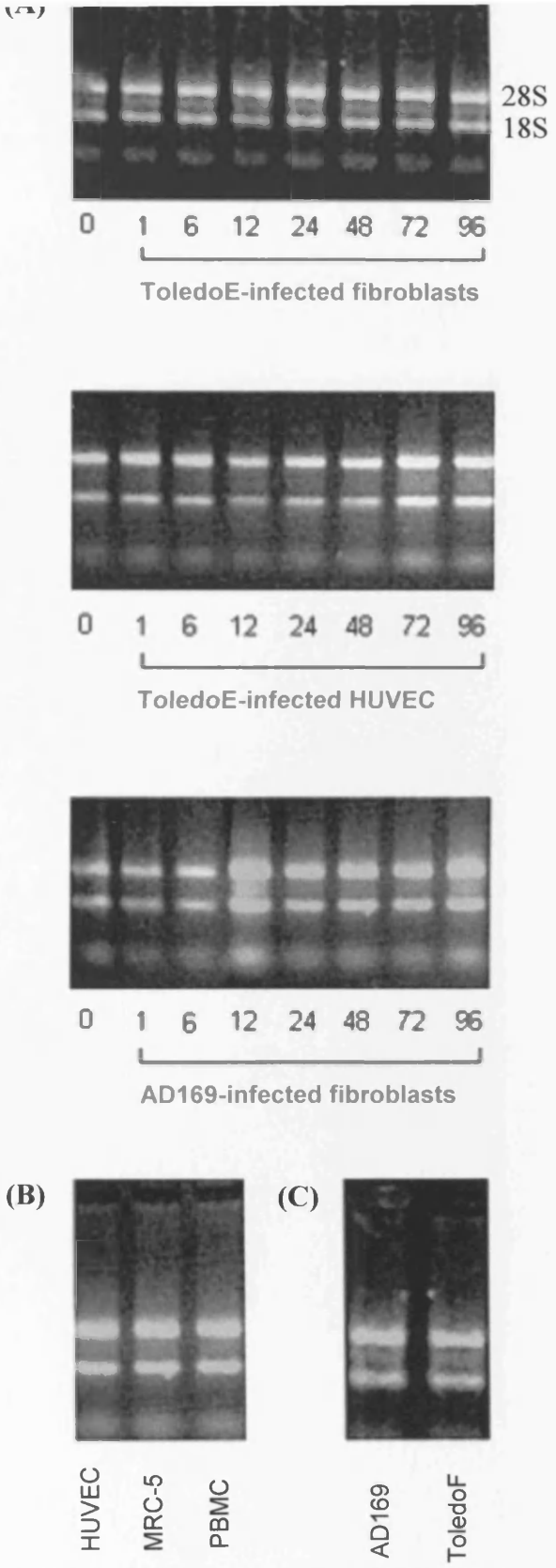


Figure 5.1. Purified total RNA. **(A)** Total RNA was quantified by UV spectrophotometry and 1µl separated by agarose gel electrophoresis. The position of 28S and 18S ribosomal RNA is indicated. The numbers indicate hours after infection. **(B)** Composition of reference RNA. **(C)** RNA extracted from fibroblasts infected with AD169 and ToledoF.

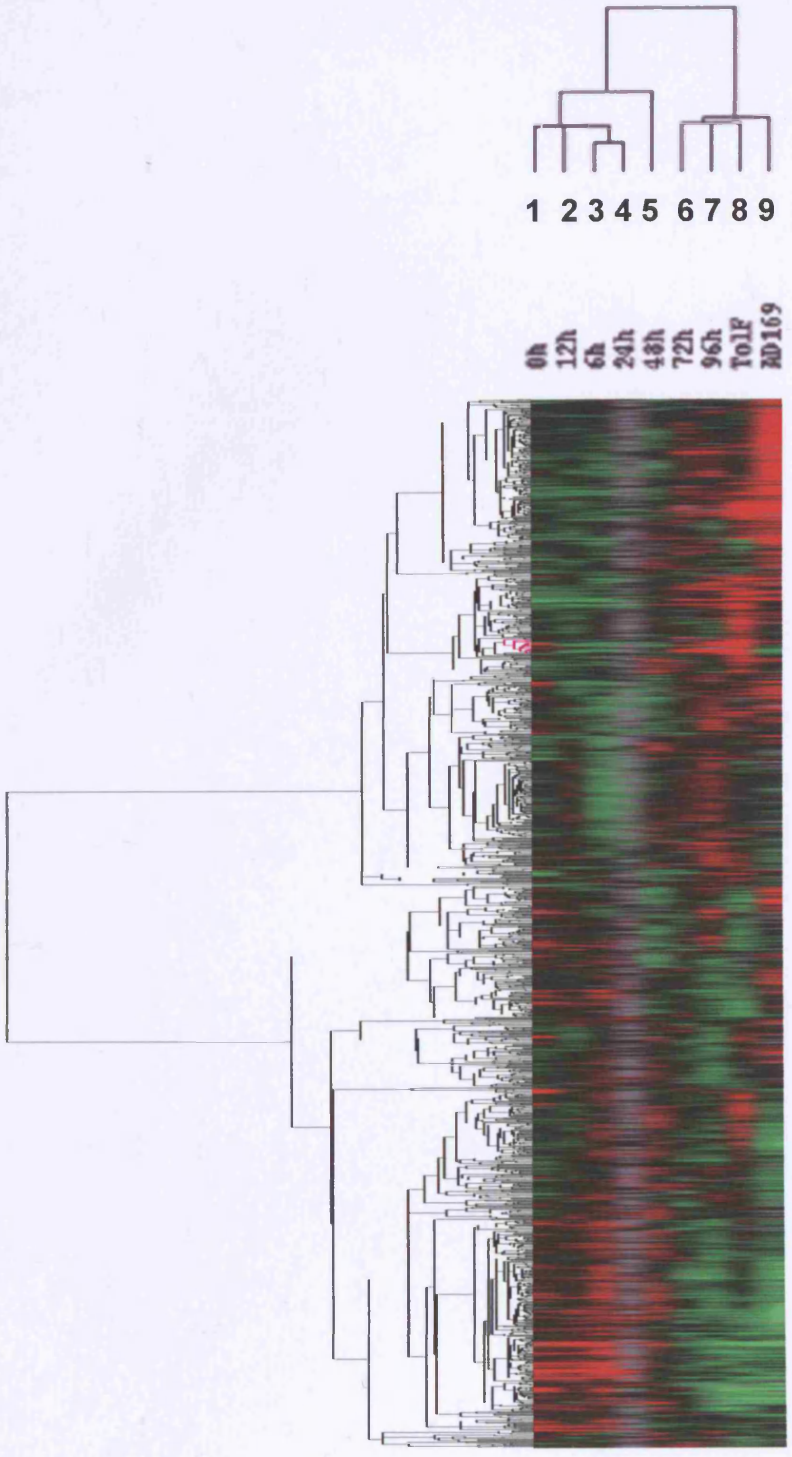


Figure 5.2. Hierarchical clustering of gene expression data. Each column represents the results from one array and each row one gene. Columns 1 to 7 show fibroblasts at successive time points in hours post infection with HCMV ToledoE. Columns 8 and 9 show fibroblasts infected with ToledoF and AD169, respectively.

5.2.3 Gene expression programmes in fibroblasts and HUVEC

Gene expression of HCMV-infected cells was analysed at several time points after infection with Toledo (0, 6, 12, 24, 48, 72 and 96 hours). The programme TreeView (Eisen *et al.*, 1998) was used to visualise the expression of the filtered set of 1746 genes (Figure 5.3) to reveal how expression of the genome varies throughout the time-course of HCMV infection and how gene expression in infected fibroblasts relates to that in infected endothelial cells. The analysis resulted in two clusters of gene expression, showing a clear distinction between the two time-courses and cell types (Figure 5.3). In turn, within each time-course, a separation between the early and late time points is evident. The two last branches of the tree (columns 8, 9) are composed of late stage cells infected with ToledoF and AD169, respectively. Their clustering indicates they have similar gene expression patterns compared to fibroblasts and endothelial cells infected with ToledoE. Overall, the clustering shows that at late times after infection (96h), all cell types, irrespective of infecting virus, look similar in terms of host gene expression. This is not surprising, since at that point cells are producing virus and dying. At 96 hours after infection, a decrease in the amount of cellular mRNAs hybridising to the arrays was observed. Earlier time points are cell type specific, showing that up to 72 hours the virus manipulates each cell type specifically.

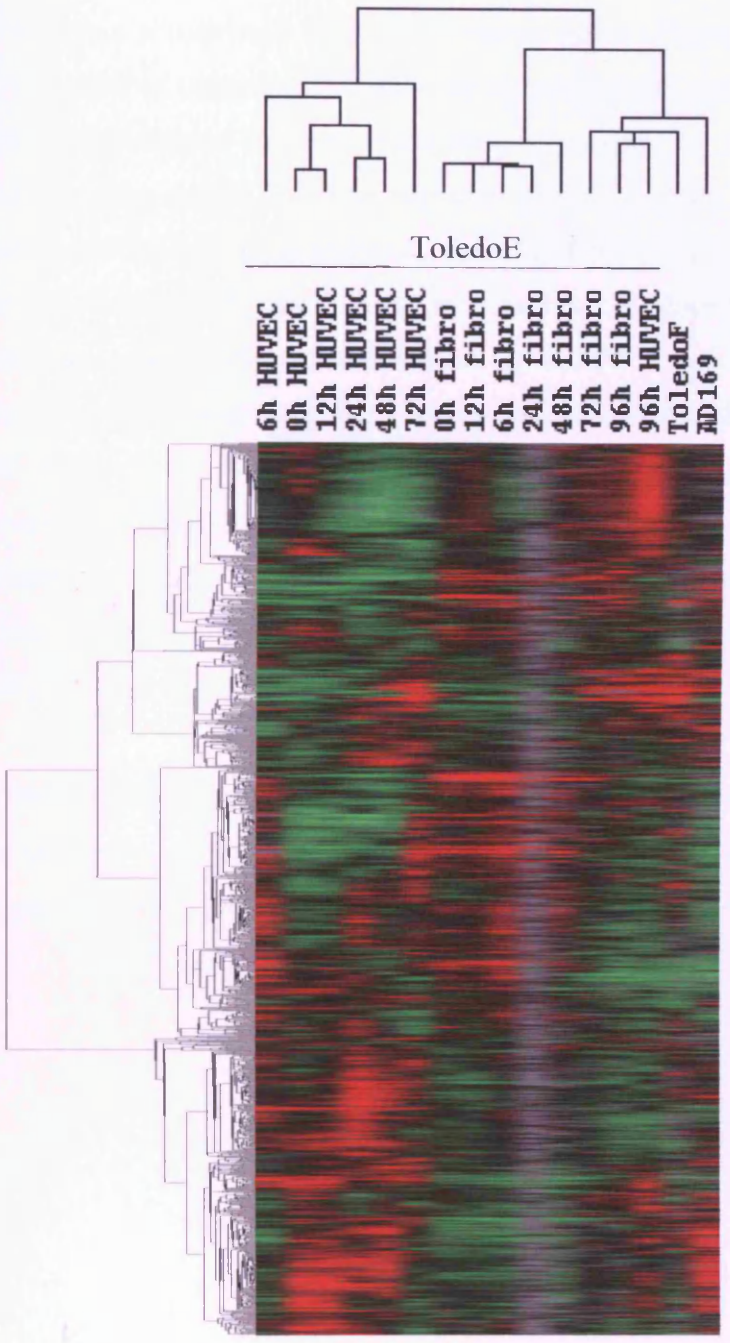
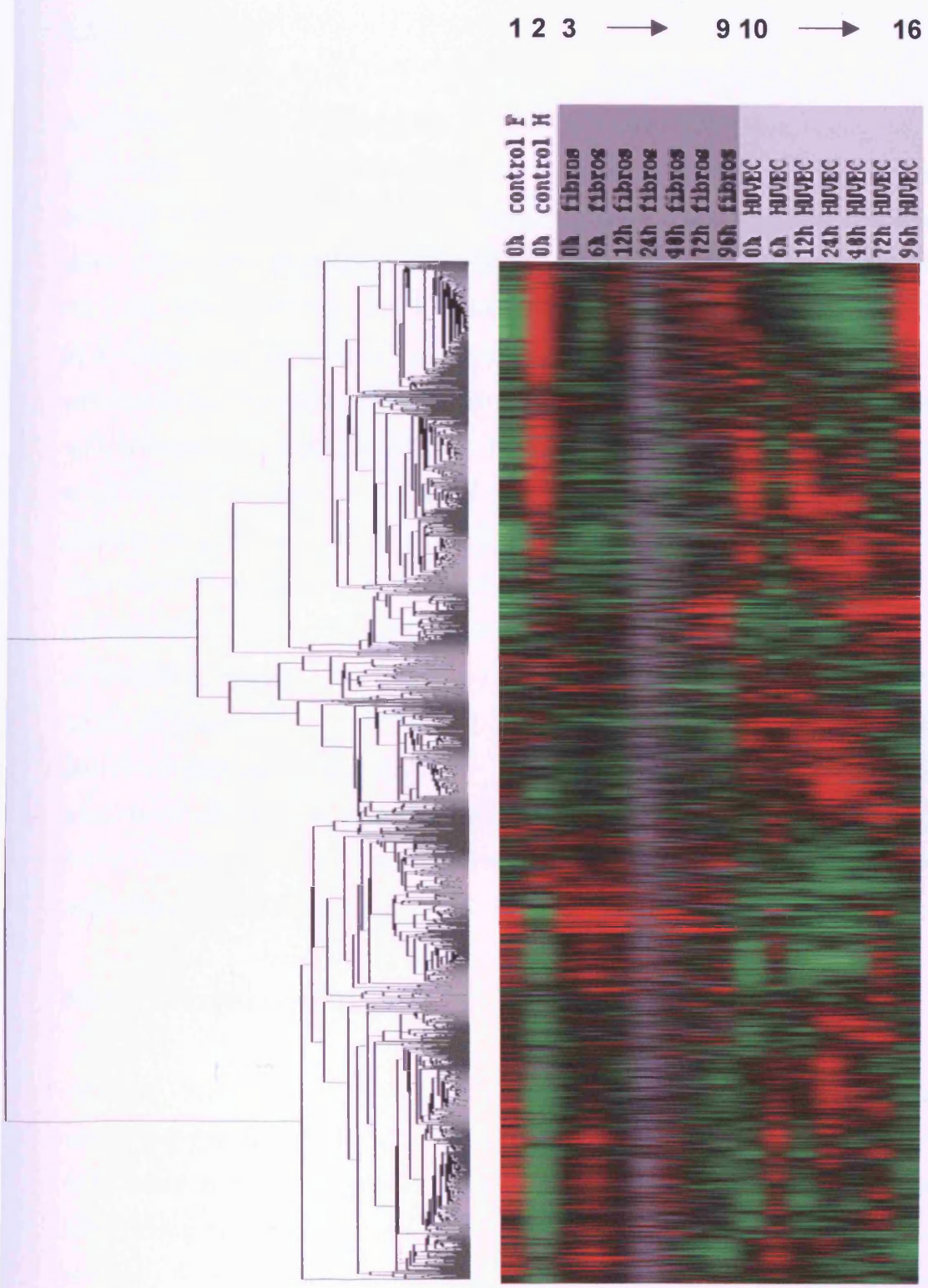


Figure 5.3. Hierarchical clustering of 16 samples and a filtered set of 1746 genes. The dendrogram on the left represents the relationship between genes in terms of their expression pattern. Gene expression is shown as a pseudo-coloured representation of log(2) expression ratio with red being above and green below the row/column median level of expression (set to 0) as shown by the scale. Columns 1 to 14 show fibroblasts and HUVEC at various time points in hours post infection with HCMV ToledoE. Columns 15 and 16 show fibroblasts infected with ToledoF and AD169, respectively.

In order to aid interpretation I used knowledge of the "domain" to order the arrays by time and cell type, producing a set of 1909 human genes (Figure 5.4). The genes and samples were ordered using a self-organising map algorithm and subjected the genes to hierarchical clustering. This revealed how expression of the genome changes throughout the time-course of infection with HCMV Toledo. A number of gene expression signatures can be identified in the data. Clustering of data from the time-courses in fibroblast and endothelial cells indicated that the levels of certain classes of host genes were notably affected in response to HCMV infection. A subset of these genes has been previously, reported, to be modulated by the AD169 strain in fibroblasts. Some examples include induction of cyclin E, CD54, Toll-like receptor 2, caspase 2 and complement C1r transcripts (Browne *et al*, 2001). I observed the same transcriptional patterns in fibroblasts after infection with HCMV Toledo. Nevertheless, I found these genes to be down regulated in endothelial cells, emphasising a degree of divergence of cell responses to viral infection.

In the following sections I will investigate how the virus modulates gene expression in the two cell types and compare it to previous findings. To make this manageable, I will break down the analysis into distinct cellular processes or disease phenotypes.

Figure 5.4. Hierarchical clustering of gene expression data. Each column represents the results from one array and each row one gene. Columns 1 and 2 show uninfected fibroblasts and HUVEC. Columns 3 to 9 show fibroblasts at successive time points in hours post infection with HCMV Toledo. Columns 10 to 16 show HUVEC at successive time points post infection. The dendrogram on the left represents the relationship between genes in terms of their expression pattern. Gene expression is shown as a coloured representation of log(2) expression ratio with red being above and green being below the median level of expression.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96h fibros
0h HUVEC
6h HUVEC
12h HUVEC
24h HUVEC
48h HUVEC
72h HUVEC
96h HUVEC

5.2.3.1 Apoptosis

Apoptosis is a way of ridding the body of unwanted cells, contributing to normal physiology. Furthermore, it is an antiviral defence mechanism that the host uses to eliminate infected cells and restrict viral propagation. To overcome this response, many viruses encode proteins that prevent or attenuate apoptosis in infected cells (O'Brien, 1998). Productive HCMV infection confers resistance to apoptosis induced by a variety of stimuli such as ligation of the death receptor Fas or TNFR1 and serum withdrawal (Kovacs *et al*, 1996, Goldmacher *et al*, 1999). HCMV encodes 4 proteins with anti-apoptotic function, IE1, IE2, UL37 and UL36 (Zhu *et al*, 1995, Goldmacher *et al*, 1999, Skaletskaya *et al*, 2001). HCMV can directly modulate various cellular genes in order to prevent apoptosis in infected cells (Zhu *et al*, 1995). Previous studies have shown that infection of fibroblasts with AD169 suppresses caspases 1, 2 and 8 (Browne *et al*, 2001, Skaletskaya *et al*, 2001). Sequential activation and over-expression of caspases plays a central role in the execution-phase of apoptosis. I found caspases 4 and 7 to be dramatically down regulated by Toledo in both fibroblasts and endothelial cells (at 72 and 48h pi respectively), indicating an interference of the virus in the caspase cascade at two additional positions (Figure 5.5A). Caspase 6 levels were preferentially down-regulated in endothelial cells, indicating a specific role for this gene in endothelial cells.

5.2.3.2 Cell cycle progression

Different viruses have different effects on the host cell cycle. Array analyses have shown that this variability may be due to differential effects on host gene expression. HPV infection stimulates entry into the cell cycle through viral gene E7 (Dyson *et al*, 1989, Munger *et al*, 1989, Nees *et al*, 2000) and the up-regulation of host genes that mediate cell cycle progression (Chang and Laimins, 2000). Endothelial cells latently infected with KSHV down-regulate GADD45 and p57-KIP2, involved in cell cycle arrest in G_1, and over-express cell cycle progression genes (Moses *et al*, 2002). The up-regulation of proliferation-associated genes by HPV and KSHV may explain their oncogenic potential.

HSV-1, reovirus serotype 3, echovirus 1 and HIV-1 infections lead to cell cycle arrest at the G2/M stage. All of these viruses up-regulate GADD45 (Hobbs and DeLuca, 1999, Khodarev *et al*, 1999, Pietiainen *et al*, 2000, Corbeil *et al*, 2001, Poggioli *et al*, 2002), a cdc2 kinase inhibitor (Wang *et al*, 1999), suggesting this may be a common mechanism for virus induction of cell cycle arrest. In addition to the up-regulation of GADD45, reovirus serotype 3 up-regulates two inhibitors of cdc2 kinase activity in M-phase, and a number of mitotic spindle checkpoint genes. Furthermore, reovirus serotype 1, which does not cause cell cycle arrest, has no effect on the expression of these genes (Poggioli *et al,* 2002).

HCMV is known to cause cell cycle arrest. Quiescent fibroblasts infected *in vitro* with AD169 start synthesising several enzymes required for DNA replication. This activation however does not seem to lead to cell division, but in G1/S arrest. This virally-induced block can take place at multiple points in the cell cycle, depending on the phase of the cell cycle at which infection occurs (Jault *et al*, 1995, Biswas *et al*, 2003). It seems that the virus creates a favourable environment for its own replication, at the host's expense. A number of cyclins are regulated by HCMV, including cyclin B, D3, E2 and G1 (Salvant *et al*, 1998, Browne *et al*, 2001). I identified a number of genes that were suppressed in fibroblasts and endothelial cells at late time points after infection, that are also required for cell cycle progression (Figure 5.5B).

Cell division cycle 2 plays a key role in the control of the eukaryotic cell cycle and is essential for G1/S and G2/M phase transitions of eukaryotic cell cycle. Cell cycle progression 2 suppresses G1 arrest. The transcript levels of these genes were suppressed by 72 hours after infection in both cell types. E2F-related transcription factor was also reduced. This gene can bind DNA cooperatively with E2F family members through the E2 recognition site, found in the promoter region of a number of genes whose products are involved in cell cycle regulation or in DNA replication. The DP2/E2F complex, functions in the control of cell cycle progression, from G1 to S phase. Cyclin D1 forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity are required for cell cycle G1/S transition. This protein has been shown to interact with tumor suppressor protein retinoblastoma (Rb) with its expression regulated positively by Rb. Cyclin D1 levels were reduced by 48 and 24 hours after infection in fibroblasts and HUVEC, respectively. This reduction in

cell cycle genes coincides with increased accumulation of viral transcripts (see section 4.2.2).

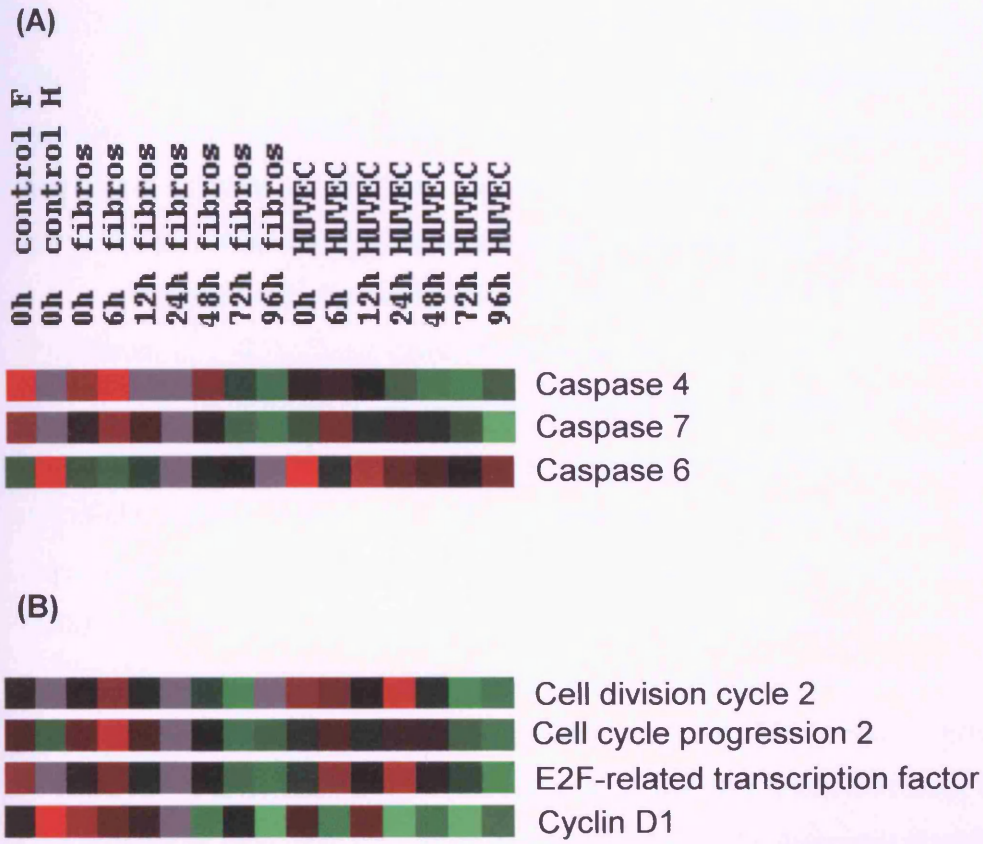


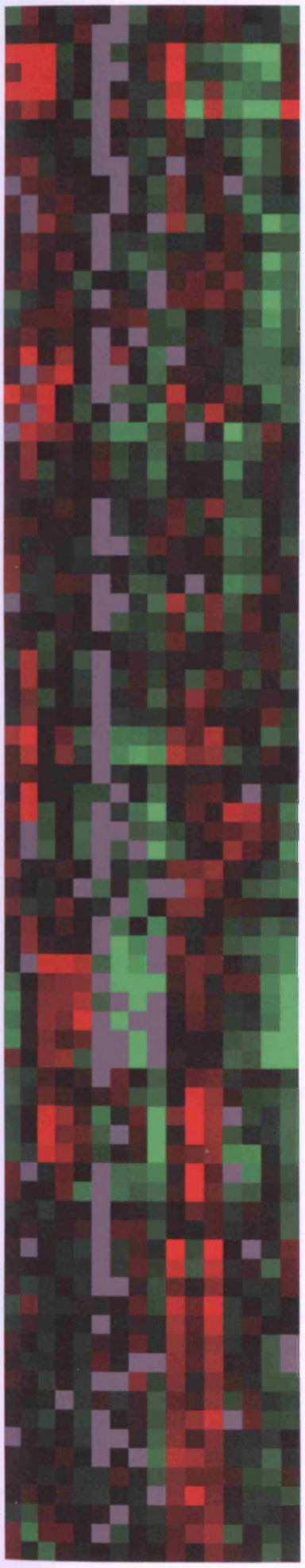
Figure 5.5. Changes in fibroblast and HUVEC gene expression profiles, induced by HCMV Toledo. mRNAs affected are involved in **(A)** apoptosis and **(B)** cell cycle progression.

5.2.3.3 Host cytoskeleton, transcription and signal transduction

Several studies indicate that viruses can induce cytoskeletal modifications in host cells (Kang *et al*, 2002, Bost *et al*, 2003, Nunbhakdi-Craig *et al*, 2003, Yang *et al*, 2003). HCMV infection causes a rapid, progressive disruption of the host cytoskeleton that correlates with actin depolymerisation. The disruption can be seen within two minutes of infection by electron microscopy (Jones *et al*, 1986). Actin appears to be the primary cytoskeletal target involved during HCMV infection, while a loss of microtubuli has also been documented within 12 hours after infection (Pfeiffer *et al*, 1983). These disruptions could facilitate viral infectivity by inhibiting the transport of host proteins that are essential for presentation of antigens to the immune system. Additionally, these early events correlate with a transient interruption of cellular mRNA translation and may represent a process whereby HCMV gene expression becomes competitive with that of the host cell (Jones and Kilpatrick, 1988). The Arp2/3 complex is required for the formation of actin filaments, presumably at the nucleation step (Higgs and Pollard, 2001, Narumiya and Mabuchi, 2002). I observed a down-regulation of ARP3, and ARP2/3 protein complex subunits p34 and p21 (Figure 5.6). This down-regulation could represent a strategy that HCMV uses to mediate its effects on actin depolymerisation and subsequent disruption of host cytoskeleton.

The cluster also contains four metallothionein genes, suggesting that there may be a common transcriptional regulatory machinery for the metallothionein genes (Figure 5.6). Metallothionein I (MT-I) and MT-II have been implicated in the protection of cells against reactive oxygen species (ROS), heavy metals, and a variety of pathological and environmental stressors. Studies have demonstrated that MT-I and MT-II can be significantly induced in the liver and lung following experimental influenza virus (Ghoshal and Jacob, 2001). A recent study showed however, that coxsackievirus B3 infection leads to suppression of metallothionein genes and increased accumulation of environmental pollutants (Funseth *et al*, 2002). These findings suggest that HCMV may interfere with the normal host responses during acute infections by down-regulating detoxifying processes in favour of acute-phase viral protein synthesis.

Figure 5.6. This cluster represents genes that are under-expressed in fibroblasts and HUVEC. The cluster contains genes that are involved in regulation of transcription and translation and in signal transduction. Lanes 1 and 2 represent uninfected fibroblasts and endothelial cells, respectively. Lanes 3 to 9 show time points after infection of fibroblasts with ToledoE, while lanes 10 to 16 show the array results from ToledoE-infected endothelial cells.



Early growth response protein 1	Transcription
GOS3	
CPBP	
HSP90	Cell cycle
CBF	Transcription
RNA pol III	
NF-AT3	
Ribosomal protein L39	Translation
MOP1	Transcription
JUN	
40S ribosomal	Translation
Myosin	Cytoskeleton
Growth factor	Signalling
M-phase inducer	Cell cycle
Cell division cycle 42	
Ribosomal protein L11	Translation
EPAS1	Transcription
Cytoplasmic dynein	
Catenin	Cytoskeleton
Latent transforming growth factor	Signalling
Metallothionein 1L	
Metallothionein-IG	Cellular defence
Metallothionein	
Metallothionein --B	
Transcription elongation factor B	Transcription
Dynein light chain 2 (LIC2)	Cytoskeleton
Ribosomal protein L27a	Translation
TRIO	Signalling
Ribosomal protein S4	
40S ribosomal protein S23	Translation
OPA-containing protein	
Pre-mRNA splicing factor	Transcription
TGF beta receptor	Signalling
Paired basic amino acid cleaving enzyme	
Translational initiation factor 2	Translation

TGF-beta (transforming growth factor beta) receptors form a heteromeric complex after binding TGF-beta at the cell surface and act as signal transducers (Hayes *et al*, 2002). The receptor for TGF-beta is down-regulated in fibroblasts, but to a further extent in HUVEC (Figure 5.6). The TGF-beta receptor is one of the substrates for the paired basic amino acid cleaving enzyme (PACE4) (Yoshida *et al*, 2001). The protein encoded by this gene belongs to the subtilisin-like proprotein convertase family. The members of this family are proprotein convertases that process latent precursor proteins into their biologically active products. PACE4 protein is a calcium-dependent serine endoprotease that can cleave precursor protein at their paired basic amino acid processing sites. My results show that TGF-beta receptor and PACE4, two functionally related genes, share the same expression profile, and therefore may be co-ordinately regulated.

5.2.3.4 HCMV disease associations

Atherosclerosis

A link between HCMV infection and atherosclerosis has been suggested by experimental, clinical and epidemiological studies (see section 1.9.5). Since endothelial cells infected with HCMV may be involved in the atherosclerotic pathway, I looked at HCMV-induced gene expression changes. I observed a loss of anti-coagulant gene expression in endothelial cells (Figure 5.7). These included members of the plasminogen subfamily. Plasminogen is the inactive precursor of the serine protease plasmin, which after activation is converted to plasmin, unfolding a potent enzymatic domain that dissolves the fibrinogen fibres that trap the blood cells in a blood clot, in a process called fibrinolysis. Plasminogen mRNA levels decreased by a factor of 9 by 24 hours after infection with HCMV. Other genes with sequence homology to plasminogen and with fibrinolytic activity were also down-regulated in infected endothelial cells, such as the hepatocyte growth factor proteins. Expression of heparan sulfate 3-O-sulphotransferase was also suppressed. Heparan sulfate is a linear polymer covalently attached to the protein cores of proteoglycans, which are abundant and ubiquitously expressed in almost all animal cells as integral membrane proteins, glycosylphophatidylinositol-linked membrane proteins, and proteins of the extracellular matrix. It assembles by the action of a large family of enzymes, with tissue-specific and developmentally regulated expression of enzyme isoforms (eg at

least four *N*-deacetylation/*N*-sulfotransferases, three 6-*O*-sulfotransferases, and five 3-*O*-sulfotransferases produce HS chains with distinct sequences) (Shworak *et al*, 1997, 1999). These different sequences enable interactions to occur with a broad array of protein ligands that modulate a wide range of biological functions in development, differentiation, homeostasis, viral entry, and coagulation cascade. Since heparan sulfate 3-O-sulfotransferase is required for the anti-coagulant properties of heparan sulfate, its suppression in HCMV-infected endothelial cells may contribute to the increased coagulation observed in infected individuals.

The virus was also shown to up-regulate the expression of two genes that contribute to the acceleration of coagulation; phospholipid scramblase, which plays a central role in the initiation of fibrin clot formation (Basse *et al*, 1996) and placental bikunin, an inhibitor of plasminogen and hepatocyte growth factor proteins (Hamasuna *et al*, 2001) (Figure 5.7). Transcript levels of the matrix metalloproteinases (MMP) 2 and 13, were reduced by 6 and 24 hours, respectively, in infected HUVEC. Several studies have documented the fibrinolytic activity of MMPs, and their essential role in neovessel formation *in vitro* and *in vivo* (Hiraoka *et al*, 1998). Finally, the MMP activators cathepsins D, H and L, were also down-regulated by 24 hours after infection. Overall, the results are compatible with previous evidence that the virus might have pro-coagulant properties (Pryzdial and Wright, 1994, Yonemitsu *et al*, 1998, Neumann *et al*, 2000). The combination of the pro-coagulant effects of HCMV infection and decreased fibrinolysis may promote the development of atherosclerosis. Additionally, CD59 was down-regulated at late time points of infection regardless of the virus strain or cell type used. CD59 is a crucial membrane complement-regulatory protein that prevents host cells from membrane attack complex (MAC)-induced cytolysis. Impaired endothelial CD59 activity may render vascular cells susceptible to MAC-induced proliferation and contribute to the development of vascular proliferative disorders.

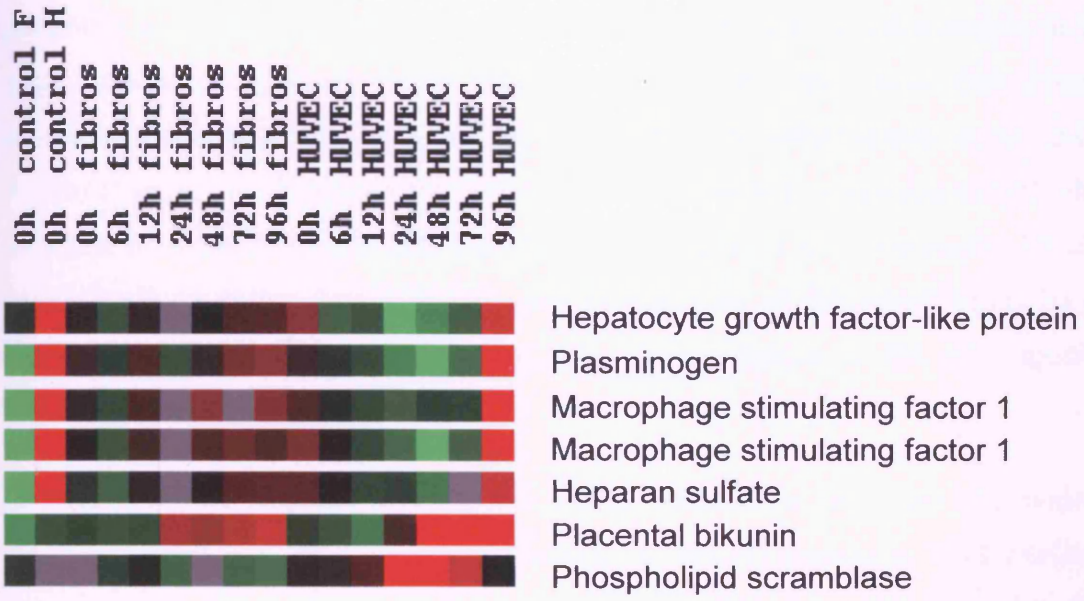


Figure 5.7. Expression of anti-coagulant genes decreases in HCMV-infected HUVEC, while the levels of placental bikunin (plasminogen inhibitor) and phospholipid scramblase (involved in fibrin clot formation) transcripts increase.

Other disease-associated genes

In the setting of immunosuppression, HCMV can affect both the central and peripheral nervous systems. Neurological manifestations of HCMV infection include encephalitis, hearing loss, mental retardation, ventriculitis, myelitis, retinitis and peripheral neuropathies (Maschke *et al*, 2002). Studies have shown that defects in the gene transforming growth factor beta-induced (TGFBI) can cause five corneal dystrophies (Fujiki *et al*, 2000, Hirano *et al*, 2001, Schmitt-Bernard *et al*, 2002, Sakimoto *et al*, 2003). My results show that TGFBI mRNA is reduced by 24h after infection of endothelial cells with Toledo HCMV (Figure 5.8). Additionally, I found genes involved in eye lens structure and transmission of the visual signal to be significantly down regulated in HUVEC after virus infection HRG4, cone-specific cGMP and crystalline genes.

Defects in the pro-a2 chain of collagen type XI (col11a2) are the cause of a skeletal dysplasia, which is accompanied by severe hearing loss (Vikkula *et al*, 1995, McGuirt *et al*, 1999, De Leenheer *et al*, 2001). The col11a2 mRNA was reduced considerably by 24h after infection with Toledo. Furthermore, defects in dihydroxyacetone (GNPAT) cause severe growth and mental retardation, retinal pigmentary degeneration, sensorineural deafness (Burdette *et al*, 1996). GNPAT mRNA levels were decreased within an hour after HCMV infection of endothelial cells (Figure 5.8). The modulation of these genes by the virus could contribute to the development of HCMV-associated diseases.

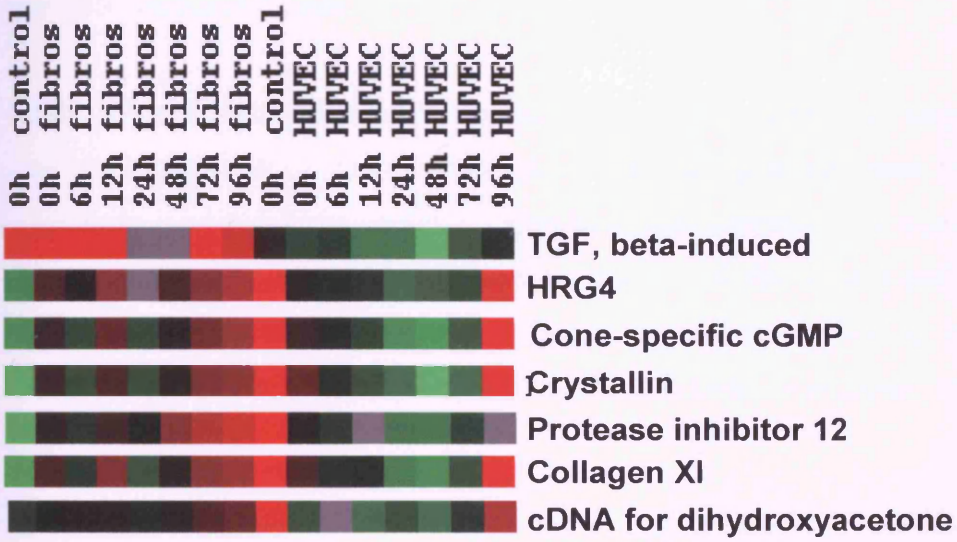


Figure 5.8. HCMV replication in HUVEC represses activation of genes that are essential for transmission of the visual signal (TGFBI, HRG4, cone-specific cGMP, crystalline) and proper functioning of the nervous system (protease inhibitor 12, collagen XI, dihydroxyacetone).

5.2.3.5 Mitochondrial genes

Studies have shown that HCMV AD169 disrupts the fibroblast reticular mitochondrial network (McCormick *et al*, 2003, Hertel *et al*, unpublished data) and stimulates mitochondrial DNA synthesis as well as lipid turnover (Furukawa *et al*, 1976). My data indicate that HCMV Toledo enhanced the mitochondrial transcription in endothelial cells 6 hours after infection, while mRNA levels in fibroblasts remained relatively unaltered (Figure 5.9).

Genes affected include mitochondrial membrane transporters and members of the respiratory chain, all of them encoded by the nuclear genome, apart from NADH-ubiquinone oxidoreductase, which is mitochondrially-encoded. The consequences of these modifications are not yet understood. One possibility is that endothelial cells have a low metabolic potential. To replicate efficiently in such an environment, HCMV may need to increase energy production in order to complete its replication cycle.

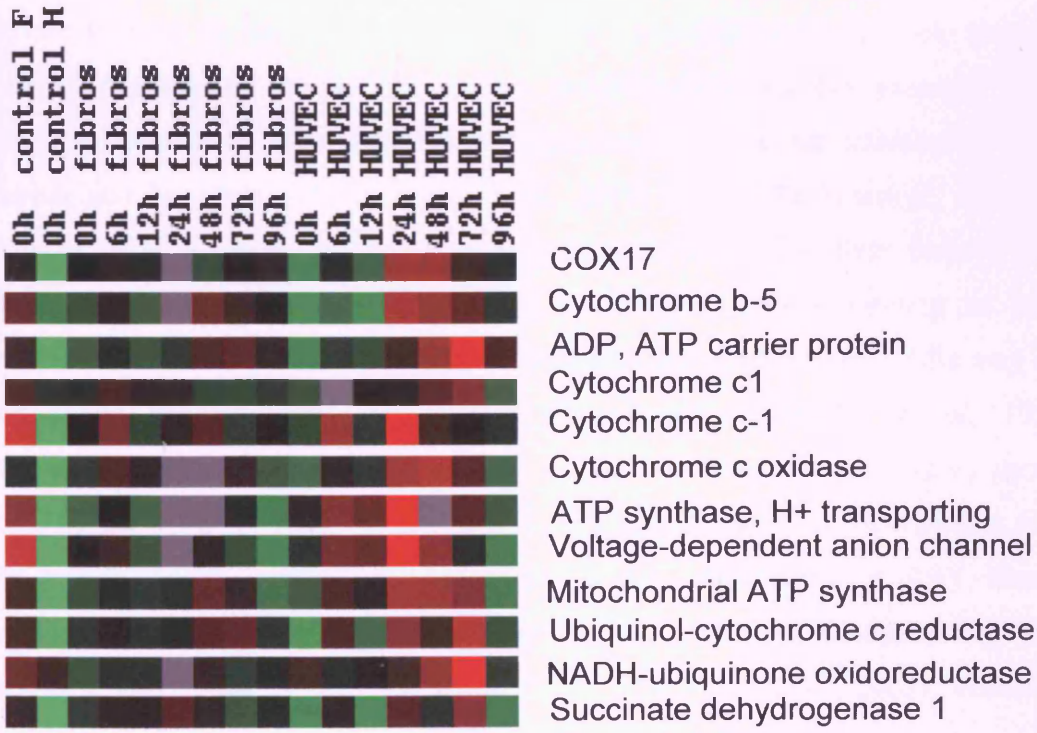


Figure 5.9. Expression of genes involved in ATP production increases in HCMV-infected HUVEC.

5.2.3.6 Immune responses

A virus capable of causing lifelong persistent infection must be able to effectively avoid the host's immune response. HCMV has developed various evasion strategies to deal with almost every immune effector mechanism (see section 1.8.3). Little is known however about the viral regulation of host immune genes in different cell types as a further strategy in immune evasion. This section presents and compares alterations to such genes, induced as a result of HCMV infection of fibroblasts and endothelial cells.

Toll-like receptors

Toll-like receptors (TLRs) are a family of ten receptors involved in microbial recognition by the immune system (Takeda and Akira, 2003). TLRs recognise a broad spectrum of ligands, including modified lipids, proteins and nucleic acids. This observed diversity in TLR ligands has raised the suggestion that different TLRs may activate different downstream responses and that these differences may help shape the immune responses to be effective against specific organisms. For example, it was recently demonstrated that TLR4 induces responses that are not activated by TLR2 (Barton and Medzhitov, 2002, Mun *et al*, 2003). All TLRs however, activate a common signalling pathway that results in the activation of nuclear factor NF-kB transcription factors (Barton and Medzhitov, 2003), thereby generating an innate immune response. Apart from recognising bacteria and plant viruses, TLRs may also function in the recognition of viruses in mammals (Whitham *et al*, 1994). Experimental evidence has shown that the respiratory syncytial virus (RSV) persists longer in the lungs of Toll-like receptor 4 (TLR4)-deficient mice (Kurt-Jones *et al*, 2000), indicating the importance of TLR4 in the pathogenesis of RSV disease. Additionally, vaccinia virus may evade host immune responses by suppressing TLR-dependent intracellular signalling (Bowie *et al*, 2000, Harte *et al*, 2003). Therefore, activation of TLRs may be involved in protecting the host from virus infection.

It has recently been shown that mere cell contact by HCMV particles leads to profound modulation of cellular gene expression, including induction of inflammatory cytokines and interferon-stimulated genes characteristic of innate immune detection

(Simmen *et al*, 2001). These findings suggested that a membrane receptor recognizes a HCMV envelope protein(s), leading to innate immune activation. Indeed, recent studies by Compton *et al* demonstrated that TLR2 recognizes HCMV virions and triggers inflammatory cytokine production, mediated via TLR2-dependent activation of NF-kB (Compton *et al*, 2003). Interestingly, I observed a suppression of TLR2 transcription by a factor of 9, 12h after infection of endothelial cells but not fibroblasts. A decrease in TLR2 levels in response to HCMV infection could reflect the shutdown of the NF-kB pathway. Consistent with a suppression of the activation of the NF-kB pathway in endothelial cells, I observed a down-regulation of several other molecules involved in the activation of this pathway (interleukin-1 receptor associated kinase, TNF type 2 receptor associated protein, TNF receptor 2 and cell death protein RIP) (Cusson *et al*, 2002, Jiang *et al*, 2002, Koay *et al*, 2002). Recent studies have also shown that HCMV infection of monocytic cell lines resulted in a reduction in cell surface expression of TNF-alpha receptor (TNFRI) (Baillie *et al*, 2003). Therefore, it seems that HCMV infection prevents external signalling to the cell in order to optimise the cellular environment for virus replication.

Interferon response and inflammation

Following infection, mammalian cells initiate a transcriptional program in order to create an antiviral response. This is usually embodied by the synthesis and secretion of type I and II interferons (IFN). Infection by EBV, KSHV, Marek's disease virus, rhesus rotavirus, measles virus, RSV, pneumonia virus of mice (PVM), hepatitis C virus (HCV) and HIV-1 all lead to an increase in the expression of interferon-stimulated genes (Bigger *et al*, 2001, Browne *et al*, 2001, Corbeil *et al*, 2001, Morgan *et al*, 2001, Bolt *et al*, 2002, Cuadras *et al*, 2002, Domachowske *et al*, 2002, Poole *et al*, 2002, Tian *et al*, 2002), indicating the activation of the cellular anti-viral response (Goodbourn *et al*, 2000). The function for interferon responsive genes in the anti-viral response is further supported by array analysis showing that the induction of interferon-responsive genes in the liver during HCV infection of a chimpanzee correlates with the clearance of viraemia (Bigger *et al*, 2001). Some viruses are able to suppress the transcriptional induction of host anti-viral genes, such as HPVs (Chang and Laimins, 2000, Nees *et al*, 2000). Similarly, there is no substantial induction in the expression of interferon responsive genes upon HSV-1 and influenza

virus infection (Geiss *et al*, 2001, Mossman *et al*, 2001). Array data therefore suggest that HSV-1, influenza virus and HPV all express genes that prevent the host anti-viral response at the transcriptional level.

Studies have shown that while the AD169 strain of HCMV inhibits IFN signal transduction in fibroblasts, it also activates some IFN-responsive genes (Zhu *et al*, 1998). The number of interferon-response genes up-regulated by UV-inactivated HCMV is also greater than for transcriptionally active virus (Browne *et al*, 2001). Treatment of cells with HCMV gB alone results in the induction of the host interferon-response genes, suggesting that gB binding is responsible for this up-regulation (Simmen *et al*, 2001). I found that the low passage strain of HCMV, Toledo, strongly induces the accumulation of IFN-responsive RNAs very early during its replication cycle in endothelial cells (6-12h pi) (Table 5.1). Interestingly, the induction is weak and delayed in fibroblasts.

One cluster of genes that are strongly induced in endothelial cells after HCMV infection are those with known anti-viral properties or that are induced by interferon (Figure 5.10A). The anti-viral cluster contains genes that act within cells to inhibit virus replication, such as IRF-1, IFI27 and STAT1 (Samuel, 2001). The interferon-induced 17 kD protein (interferon stimulated gene (ISG) 15), which is released upon virus infection (Cook *et al*,. 1995) and interferon-inducible protein 1-8U are also up-regulated strongly in endothelial cells, as indicated by my data. The virus also increases the expression levels of two genes that are transcribed in response to IFN-gamma, namely death-associated protein kinase 1 (Cohen *et al*, 1997, Inbal *et al*, 1997, Chawla-Sarkar *et al*, 2003) and NMI (Zhu *et al*, 1999, Zhou *et al*, 2000, Chen and Naumovski, 2002). Genes responsible for antigen presentation are also contained within the anti-viral cluster, such as MHC class I and tapasin, a protein involved in the association of MHC class I with transporter associated with antigen processing (TAP) and in the assembly of MHC class I with peptide (Momburg and Tan, 2002, Garbi *et al*, 2003, Vilches, 2003) (Figure 5.10B). Overall, my results indicate that the virus affects IFN signalling differentially in fibroblasts and endothelial cells, inducing a vigorous response in the latter cell type. This could be a tactic that HCMV employs in order to reduce its replication levels, facilitating its long-term association with the host.

The same cluster contains four inflammatory genes, proteoglycan 1, CD9 antigen, leukotriene A4 hydrolase and microsomal glutathione S-transferase (Broberg *et al*, 2002, Haeggstrom *et al*, 2002, Sherratt *et al*, 2003, Zaitsu *et al*, 2003) (Figure 5.10C). The latter two genes mediate prostaglandin synthesis. It has been shown that the synthesis of prostaglandin E2 is activated by the induction of cox-2 in HCMV-infected fibroblasts (Zhu *et al*, 1998). Furthermore, experiments using cox-2 inhibitors in fibroblast cultures showed that there is a dose-dependent inhibition of HCMV growth *in vitro*. It therefore seems likely that the virus requires this pathway to complete its replication cycle. We observed increased accumulation in prostaglandin synthesis in endothelial cells compared to fibroblasts, occurring at just 1 hour after infection (leukotriene A4 hydrolase, microsomal glutathione S-transferase) (Figure 5.10C). This could have significant clinical applications, since it suggests that inhibitors may have differential effects in different cells in the body.

(A)

(B)

(C)

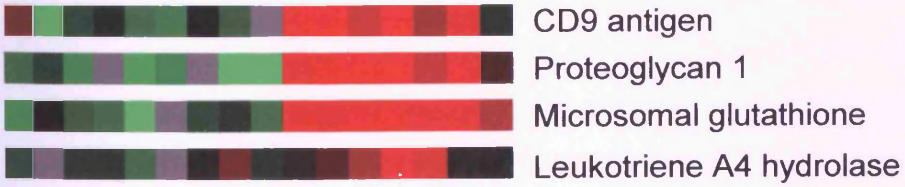


Figure 5.10. Induction of immune response genes after HCMV infection of fibroblasts and HUVEC.

MHC-II and complement

One of the major mechanisms of immune recognition that HCMV must overcome is presentation of antigens by MHC class II to stimulate the adaptive immune response. A number of reports indicate that HCMV interferes with both MHC class I and II pathways of antigen presentation (Gil-Torregrosa *et al*, 1998). Under normal circumstances, IFN-gamma is a potent inducer of MHC class II expression in antigen presenting cells and other cell types such as endothelial and epidermal cells (Fritchley *et al*, 2000, Skovseth *et al*, 2002). However, in HCMV-infected cells, IFN-gamma is unable to induce the production of MHC class II mRNA, suggesting an interference with the Jak/Stat signal transduction pathway (Sedmak *et al*, 1994, Miller *et al*, 1998). There is also considerable evidence that HCMV may disrupt MHC class II expression in infected monocytes (Moutaftsi *et al*, 2002). My results show that the virus induces a sharp shutdown in MHC-II transcription in endothelial cells within an hour after infection and lasting for 72h pi, possibly preventing a full cytotoxic T cell (CTL) response by modulating the T helper response and leading to persistence of the virus in the body (Figure 5.11).

Another arm of the immune system that HCMV must combat is the humoral response. Viruses may be eliminated from the body via complement-mediated lysis of infected cells. HCMV infection increases the fibroblast cell surface expression of two proteins, membrane cofactor protein (or CD46) and decay accelerating factor (CD55), two members of the regulator of complement activation (RCA) gene cluster, by up to eightfold (Spiller *et al*, 1996). Under normal conditions, these proteins protect host cells from complement-mediated cell lysis by inhibiting C3 convertases (Lindahl *et al*, 2000, Miwa and Song 2001). The role of CD55 in protection of HCMV virions against complement-mediated cell lysis was established by stripping virions of CD55 and showing that these virions were more susceptible to lysis, while reconstitution of CD55 restored resistance to lysis (Spear *et al*, 1995). My analysis showed that HCMV Toledo induces up-regulation of CD46 and CD55 in both fibroblasts and HUVEC, but to higher levels in the latter cell type. Additionally, the complement components 1, C2 and 8 were suppressed at 6h after infection only in HUVEC (Figure 5.11). Overall, the inhibition of the complement cascade was more prominent in endothelial cells compared to fibroblasts. The down-regulation of complement proteins in endothelial

cells has not been shown before and represents a unique strategy of HCMV for modulation of the complement cascade. The circumvention of complement attack can lead to persistence of HCMV-infected endothelial cells in the circulation and subsequent spread of the virus to different sites in the body.

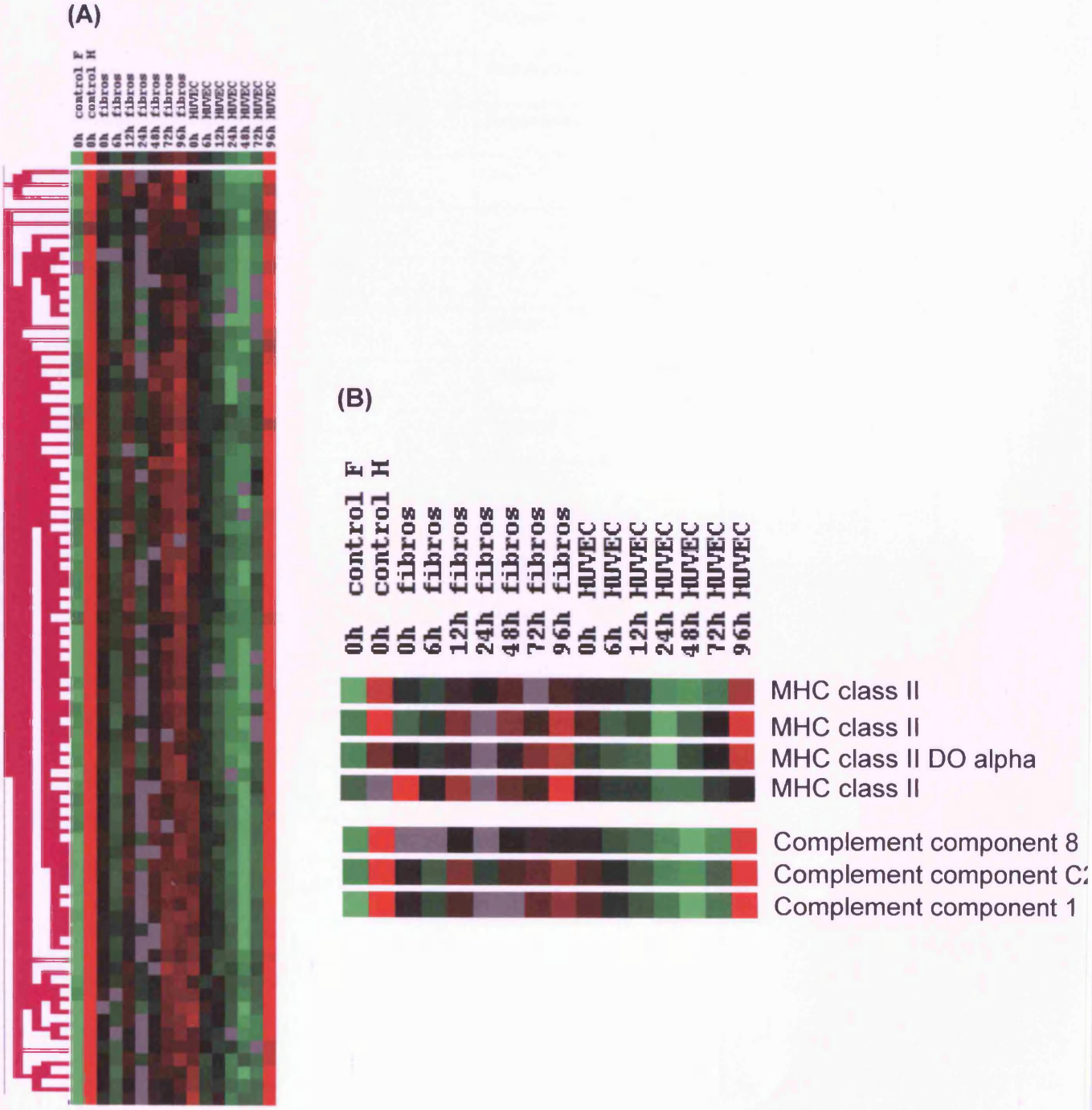


Figure 5.11. (A) Different cellular transcriptional responses to HCMV Toledo infection using DNA microarrays. When HUVEC and fibroblasts were infected, differential gene expression occurred in the two cell types. **(B)** Detail of the cluster. Infection of HUVEC results in down regulation of MHC class II and complement genes.

Gene	Change of gene expression in fibroblasts	Change of gene expression in HUVEC
Cell cycle genes		
Cyclin E	Induced	Suppressed
Cell division cycle 2	Suppressed	Suppressed
Cell cycle progression 2	Suppressed	Suppressed
E2F-related transcription factor	Suppressed	Suppressed
Cyclin D1	Suppressed	Suppressed
Interferon-responsive genes		
IFN-inducible protein 1-8U	Induced	Induced strongly
IFN-induced 17kD	Induced	Induced strongly
Interferon regulatory factor 1 (IRF1)	Induced	Induced strongly
IFI27	Induced	Induced strongly
DAP-kinase	Induced	Induced strongly
NMI	Induced	Induced strongly
Tapasin (NSG-17)	Induced	Induced strongly
Apoptotic genes		
Caspase 4	Suppressed	Suppressed
Caspase 6	Induced	Suppressed
Caspase 7	Suppressed	Suppressed
GT197 partial ORF	Suppressed	Suppressed
E1B 19K/Bcl-2-binding protein Mip3	Suppressed	Suppressed
Complement pathway genes		
Complement C1r	Induced	Suppressed
Complement C2	Induced	Suppressed
Complement 8	Induced	Suppressed
CD46	Induced	Suppressed
CD55	Induced	Suppressed
Prostaglandin synthesis genes		

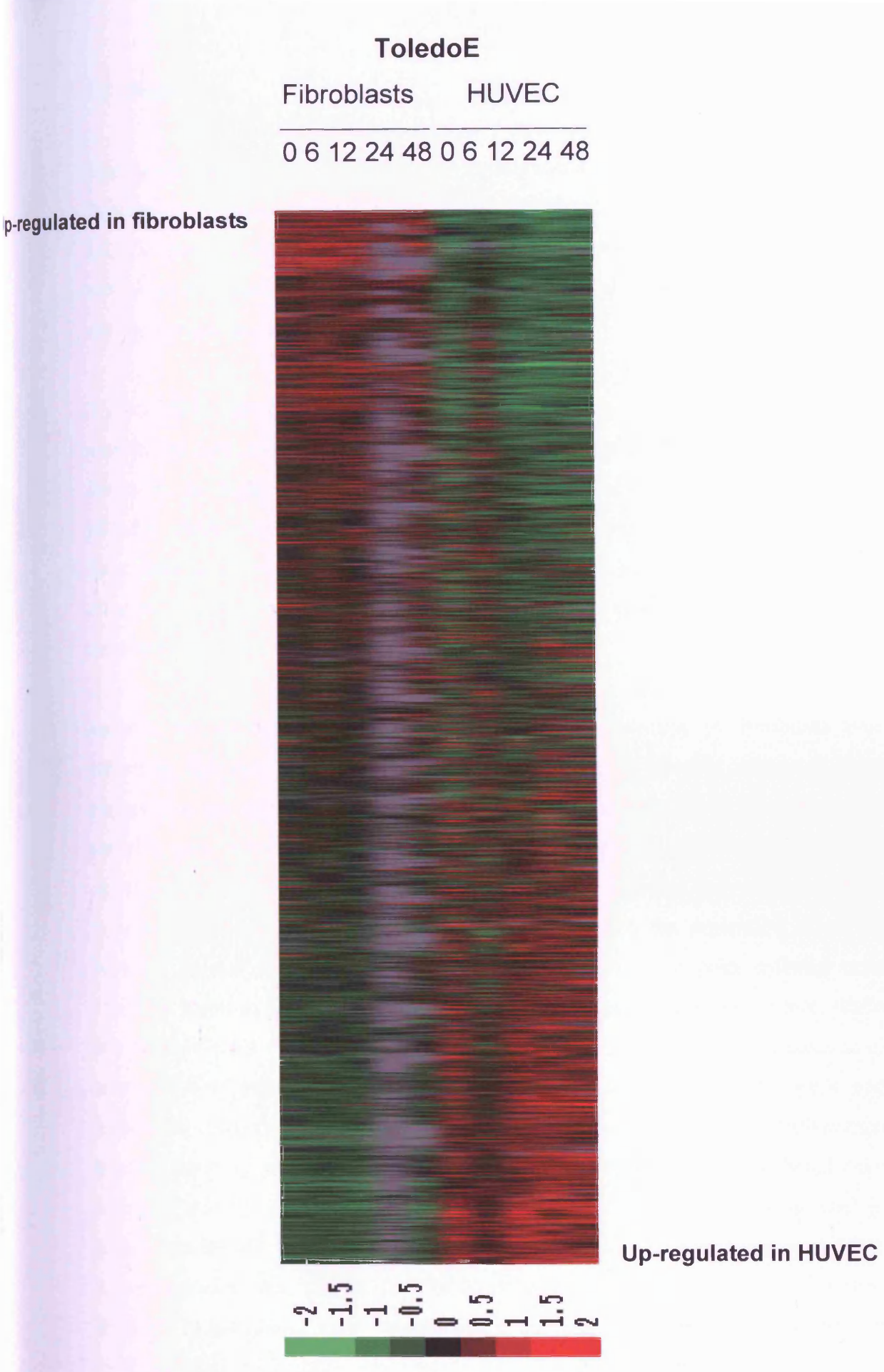
Leukotriene A4 hydrolase	Induced	Induced strongly
Microsomal glutathione transferase	Induced	Induced strongly
Mitochondrial genes		
COX17	Suppressed	Induced
Cytochrome b-5	Induced	Induced
NAD-dependent methylene	Induced	Induced
ADP, ATP carrier protein	Suppressed	Induced
Cytochrome c1	Suppressed	Induced
Cytochrome c-1	Suppressed	Induced
Cytochrome c oxidase	Suppressed	Induced
ATP synthase, H+ transporting	Suppressed	Induced
Voltage-dependent anion channel	Suppressed	Induced
Mitochondrial ATP synthase subunit	Suppressed	Induced
Ubiquinol-cytochrome c reductase	Suppressed	Induced
NADH-ubiquinone oxidoreductase	Suppressed	Induced
Succinate dehydrogenase 1	Suppressed	Induced
Atherosclerosis-related genes		
Hepatocyte growth factor-like protein	Induced	Suppressed
Plasminogen	Induced	Suppressed
Macrophage stimulating 1	Induced	Suppressed
Heparan sulfate	Induced	Suppressed
Placental bikunin	Induced	Induced
Phospholipid scramblase	Suppressed	Induced
Disease-associated genes		
TGF beta-induced	Suppressed	Suppressed
Protease inhibitor 12	Induced	Suppressed
Collagen XI	Induced	Suppressed
Dihydroxyacetone	Unchanged	Suppressed

Table 5.1. Representative list of cellular RNAs modulated as a result of HCMV infection of fibroblasts and HUVEC.

5.2.3.7 Mann-Whitney test

The Mann-Whitney U test is a non-parametric version of the t-Test, has been used in a number of array studies to assess the stastical significance of differential gene expression (Chambers *et al*, 1999, Hedenfalk *et al*, 2001, Notterman *et al*, 2001, Zhan *et al*, 2002). Using this technique, the differential gene expression of many genes in fibroblasts and HUVEC was found to be highly statistically significant ($p<0.005$) (Figure 5.12). This does not detect differences because of virus infection, but illustrates constant cell type specific differences. The expression levels of some of these genes remained relatively constant within the 48 hours of infection in each cell type. This is supporting of the fact that a number of cellular RNAs are expected to remain unchanged even after virus infection. Genes that are expressed at very low levels in HUVEC include ones involved in extracellular matrix formation and cell adhesion, such as alpha-3 collagen, tenascin C, procollagen-proline, CDH3, CD44, fibronectin 1, laminin, and collagen binding protein 1 (Erickson, 2002, Duguay *et al*, 2003, Hirano *et al*, 2003). The bottom part of the Mann-Whitney diagram includes genes that are expressed at high levels in HUVEC. This cluster is mainly composed of genes that are part of the antiviral response to infection, such as inflammatory genes (TNF-alpha stimulated ABC protein, azurocidin 1) (Richard *et al*, 1998, Edens and Parkos, 2003) and interferon-inducible genes (DAP-kinase, Nmi mRNA).

Figure 5.12. Ordering of 1990 genes using a Mann-Whitney U test, comparing gene expression differences between fibroblasts and endothelial cells. Each column represents one sample and each row one gene.


ToledoE
Fibroblasts
HUVEC
0 6 12 24 48 0 6 12 24 48
p-regulated in fibroblasts
Up-regulated in HUVEC
-2
-1.5
-1
-0.5
0
0.5
1
1.5
2

5.3 Discussion

Regulation of infection and *in vivo* pathogenesis involves multiple points of interaction between viral and host factors. Deciphering the mechanisms by which HCMV interacts with the cell can increase our understanding of the basic biology of virus-host interactions, uncover genes whish are essential in pathogenesis and help to create appropriate strategies to combat disease.

To date, there have been no reports comparing the effects of HCMV in fibroblasts and endothelial cells, two cell types that clearly play a pivotal role *in vivo* during HCMV infection. In addition, most studies have used the laboratory-adapted strains of HCMV, which may not be fully representative of the wild type virus. In this study I have identified similarities and differences in viral and host transcriptional patterns after infection of these two cell types with a low passage strain of HCMV, using DNA microarrays.

Initially, the arrays were used to monitor host gene expression in fibroblasts after infection. The data were examined by cluster analysis to identify patterns in gene expression and identify relationships between the samples. Both the genes and samples were ordered using a self-organising map algorithm, resulting in two major clusters of gene expression. This revealed a separation between early and late events in viral replication at the level of the host genes, with the separation occurring between 24 and 48h after infection (Figure 5.2). Early time point infected cells clustered together, indicating that they share similar gene expression pattern, while late stage-infected cells formed a separate cluster. To assess and compare patterns of host and viral gene expression during HCMV infection in both fibroblasts and endothelial cells, the data were combined and filtered to produce a set of 1909 human genes. Clustering of data from the time-courses in fibroblast and endothelial cells indicated that the levels of certain classes of host genes were notably affected in response to HCMV infection. A subset of these genes has been previously, reported to be moderated by the AD169 strain in fibroblasts. I found these genes to be down-regulated in endothelial cells, emphasising a degree of deviation of cell responses to viral infection. Comparative microarray analysis of VZV-infected T cells, fibroblasts and skin has also demonstrated many differences in the response phenotype of host

cell genes in each gene type, including ones involved in signal transduction and apoptosis (Jones and Arvin, 2002). Common pathways of host gene expression were identified in fibroblasts and endothelial cells, including a shutdown of signal transduction, an inhibition of apoptosis and cell cycle progression, an increase in overall MHC class I transcription and the induction of an interferon-based antiviral response. Nevertheless, cell-type specific responses to HCMV infection were also observed, including increased pro-thrombotic activity, prostaglandin synthesis and the induction of mitochondrial-associated genes in endothelial cells but not fibroblasts. Surprisingly, immune responses were suppressed to a further extent in endothelial cells compared to fibroblasts.

The majority of genes modulated by the virus include ones that mediate host immune responses to infection. One reason that HCMV carries a great abundance of genes is that this collection provides the virus with ways to respond to different host immune control. Innnate and adaptive immune clearance presents a clear and present danger to the virus and is the target of virus immune regulation. Interestingly, a dramatic suppression of TLR2 RNA expression was observed after infection of endothelial cells but not fibroblasts. This decrease in TLR2 levels could inhibit the NF-kB mediated inflammatory response and subsequently lead to viral persistence in endothelial cells. Consistent with these findings, HCMV infection also circumvents the NF-kB pathway in retinal pigment epithelial (RPE) cells (Cinatl *et al*, 2001). This circumvention could explain the pathological features of HCMV in the eye, such as low inflammatory responses, that lead to the development of HCMV retinitis.

It has been shown that the virus also interferes with antigen presentation, apoptotic mechanisms and the complement cascade. My results show that the virus induces a rapid decrease in MHC class II expression in endothelial cells within an hour after infection and lasting for 72h pi. This would inhibit the presentation of viral antigens to T helper cells, altering CD8 T cell responses and leading to an environment conducive to the maintenance of the virus in the body (Figure 5.11). Furthermore, a strong inhibition of the complement cascade was observed in endothelial cells, and three novel genes in this cascade that the virus manipulates were identified, namely complement 1, C2 and 8 (Figure 5.11). This escape from complement attack can lead to persistence of HCMV-infected endothelial cells in the circulation. Another way by

which HCMV can achieve this persistence is by inhibiting the apoptosis of infected cells. Indeed, in our experiments, caspases 4 and 7 were found to be significantly down regulated by Toledo in both fibroblasts and endothelial cells, indicating an interference of the virus in the caspase cascade at two additional positions (Figure 5.5A). All the strategies that HCMV employs can lead to persistence of infected cells in the circulation and subsequent spread of the virus to different sites in the body.

A startling feature of HCMV infection is how the host can be tricked into committing its immune response to aiding virus dissemination. For example, the results in this chapter indicate that the virus affects IFN signalling differentially in fibroblasts and endothelial cells, mounting a vigorous response in the latter cell type. This could be a tactic that HCMV employs in order to reduce its replication levels, facilitating its long-term association with the host. Previous studies suggested that HCMV requires the synthesis of prostaglandins to maintain its growth in fibroblasts, since the use of prostaglandin inhibitors reduced replication levels in culture. I found a stronger up-regulation of these genes in endothelial cells compared to fibroblasts, implying that inhibitors of prostaglandin synthesis may have differential effects in different cells in the body and careful consideration must be taken before their appliance. Therefore, this comparison of gene expression levels in two different cell types can potentially have significant clinical applications.

Epidemiologic data and pathologic studies suggest a link between HCMV infection and atherosclerosis. My results showed a down-regulation of anti-coagulants and genes involved in fibrinolysis, consistent with previous evidence that the virus might have pro-coagulant properties (Pryzdial and Wright 1994, Yonemitsu *et al*, 1998, Neumann *et al*, 2000). Additionally, the levels of CD59, a complement inhibitor, were suppressed in cells after HCMV infection. The combination of the pro-coagulant effects of HCMV infection, decreased fibrinolysis and MAC-induced proliferation may contribute to the development of vascular proliferative disorders.

HCMV has been found in many areas of the body including the lungs, GI tract, oesophagus and most commonly the retina. Not only is retinal infection by HCMV the most common site for the virus to manifest itself, it is also the most common opportunistic infection seen in patients with AIDS. Although most patients are

asymptomatic, retinitis continues to be a chronic ophthalmologic problem among HIV-1-infected patients who do not respond to highly active anti-retroviral therapy, with symptoms ranging from floaters to dramatic vision loss. Although HCMV retinitis occurs during HIV-1-induced immunosuppression, the precise mechanisms that fail during the immunopathogenesis of AIDS to allow onset and progression of HCMV retinal disease remain undefined. Additionally, studies have failed to explain how HCMV gains access to and initiates infection of the retina. In eyes with HCMV retinitis, viral presence has been demonstrated in glial, retinal pigment epithelial, neuronal, and endothelial cells (Read *et al*, 1999). A model of HCMV retinal infection has been proposed, suggesting that infection of endothelial cells leads to infection of surrounding glial and neuronal cells, with eventual spread to the RPE cells.

A study by Brody *et al* looking at 21 AIDS patients with HCMV retinitis showed that 90% of these patients contained corneal endothelial deposits (Brody *et al*, 1995). Studies have demonstrated that defects in transforming growth factor beta-induced gene (TGFBI) can cause 5 corneal dystrophies (Fujiki *et al*, 2000, Hirano *et al*, 2001, Schmitt-Bernard *et al*, 2002, Sakimoto *et al*, 2003). My results showed a reduction in the mRNA of this gene by 24h after infection of endothelial cells with Toledo HCMV (Figure 5.8). Additionally, I found genes involved in eye lens structure and transmission of the visual signal to be significantly down regulated in HUVEC after virus infection (HRG4, cone-specific cGMP and crystalline genes) (Kubota *et al*, 2002, Fu and Liang, 2003). The suppression of these mRNAs could contribute to the development of eye abnormalities in HIV patients infected with HCMV.

In the setting of HIV infection, cytomegalovirus can affect both the central and peripheral nervous systems. Apart from retinitis, neurological manifestations of HCMV infection include encephalitis, ventriculitis, myelitis and peripheral neuropathies (Bray *et al*, 1981, Maschke *et al*, 2002). Protease inhibitor 12 may be required for the formation or reorganisation of synaptic connections as well as for synaptic plasticity in the adult nervous system. Defects in this gene are the cause of familial encephalopathy. I observed a decrease in its mRNA in endothelial cells 6h after infection, which could contribute to the development of encephalopathy observed in HIV infected patients.

HCMV is the leading viral cause of congenital abnormalities, with an incidence of 1-2.4% of live births, of whom severe classic "cytomegalovirus inclusion disease" is observed in 10%. Congenital HCMV infection is the leading infectious cause of brain damage and hearing loss in children. Defects in the pro-a2 chain of collagen type XI (col11a2) are the cause of a skeletal dysplasia, which is accompanied by severe hearing loss. The col11a2 mRNA was reduced considerably by 24h after infection with Toledo. It is possible that this decline could contribute to the development of hearing loss in foetuses congenitally infected with HCMV. Defects in the cDNA for dihydroxyacetone (GNPAT) cause severe growth and mental retardation. GNPAT mRNA levels were decreased within an hour after HCMV infection of endothelial cells (Figure 5.8). HCMV potentially could modulate GNPAT in endothelial cells, contributing to the development of mental retardation in children congenitally infected with the virus.

Detection of the host transcriptional networks that are modulated as a result of viral infection can contribute to our understanding of the mechanisms by which by which HCMV causes disease and provide the keystone for the development of novel therapeutic strategies. Microarray experiments can provide a large amount of information to stimulate further research and give clues to what cellular genes the virus needs for its replication. Since the first isolation of HCMV in 1956, this virus has been routinely passaged in fibroblast cell cultures. However, several pieces of evidence suggest that the resulting combinations of viral strains/cell lines are not faithful paradigms of disease pathogenesis in humans. First, HCMV is tropic for endothelial cells, monocytes and multiple cells of epithelial cells in origin *in vivo* leading to hepatitis, retinitis, pneumonitis, enteritis and a possible contribution to atherosclerosis. Second, these diseases occur with different frequencies in distinct groups of infected patients, implying a host contribution to viral pathogenesis. In this chapter I have reported the use of arrays to examine pathways of HCMV interaction with the cellular machinery of fibroblasts and endothelial cells. Although common profiles of cellular gene transcripts were induced or suppressed in these cell types, significant alterations in cellular gene regulation were also observed and have been focused on here, suggesting specific differences in the biological consequences of HCMV infection related to the target cell. I have also pinpointed several genes, which may be implicated in the development of HCMV disease, although these roles remain

highly speculative. However, since these genes are modulated as a result of the host-pathogen interaction, they deserve further examination. Overall, the use of microarrays to demonstrate differences in effects on host cell genes in biologically relevant cell types provides detailed information for experiments to link these various response phenotypes with mechanisms of HCMV pathogenesis that are important for the natural course of human infection.

Chapter 6
HCMV strain-specific effects on host transcription

6.1 Introduction

Gene expression changes may reveal key regulatory differences that lead to different virulence patterns between closely related pathogen strains. For example, variations in virulence of *Listeria monocytogenes* serotypes have been correlated with differential transcription of PrfA-regulated virulence genes (Bohne *et al*, 1996, Sokolovic *et al*, 1996).

Comparisons of the nucleotide sequences of AD169, Towne, Toledo and several clinical isolates of HCMV indicated that clinical isolates and Toledo contain a DNA segment composed of 19 open reading frames that are not present in AD169 and a 13kb fragment that is absent from Towne (Cha *et al*, 1996). This suggested that wild type HCMV contains genes that may be lost during repeated laboratory propagation and that these genes may contain viral determinants of cell or tissue tropism that can be lost during passage in culture. Indeed, several studies have reported growth differences between HCMV strains propagated in various cell lines. For example, it was demonstrated that clinical isolates but not AD169 or Towne could productively infect endothelial cells (Sinzger *et al*, 1997). Additionally, a HCMV clinical isolate could efficiently infect fibroblasts, smooth muscle cells and endothelial cells, whereas AD169 could efficiently infect only fibroblasts. The maintenance of these ORFs in clinical isolates suggests that the encoded gene products are important for HCMV pathogenesis *in vivo* and may represent virulence factors.

When the replication of different HCMV strains was evaluated in SCID-hu retinal tissue implants, *in vivo*, neither AD169 nor Towne replicated in the implant tissue to titres observed after infection with the Toledo strain (Bidanset *et al*, 2001). Another study showed that after infecting thymus and liver implants engrafted in SCID mice, Toledo replicated to titres that were 2-3 orders of magnitude higher than those observed for AD169 (Brown *et al*, 1995). Furthermore, when AD169, Towne and Toledo were tested in controlled human trials, AD169 and Towne were shown to be avirulent in HCMV seronegative volunteers. In contrast, the low passage strain Toledo, produced clinical disease when administered to healthy adult volunteers (Quinnan *et al*, 1984). Such studies are of particular interest, since ORFs UL133 to UL151, like other unique genes of individual herpesviruses, might only be relevant for replication in the natural host organism. Additionally, because HCMV exhibits a

very narrow host range, it is possible that these genes are adaptations that benefit replication in the human host.

Because of the putative application of AD169 and Toledo as vaccines, it is important to study and compare the interactions of these viruses with the host in detail. Recently, Northern blot analysis of 58 cellular genes revealed no differences in gene expression induced by the AD169 and Toledo strains of HCMV (Zhu *et al*, 1998). Despite these findings, it has been demonstrated that clinical isolates of HCMV induce changes in normal cell functions different or at different levels to that induced by the laboratory-adapted strains. For example, infection of HUVEC with a clinical isolate induced an increase in their proliferation, while AD169 failed to do so. The clinical isolate also affected cytokine production and the expression integrin receptors for fibronectin and laminin (Woodroffe *et al*, 1994, 1997).

A broader understanding of the transcriptional changes associated with viral infection will reveal important details on how the host responds to AD169 and Toledo. DNA microarray techniques and oligonuclaotide arrays provide sensitive and reliable methods for this purpose. In this chapter I used the DNA microarrays to identify global changes in host transcription induced by these two HCMV strains and attempted to determine the functional effects of the additional genes present in the clinical strains.

6.2 Results

6.2.1 Sample preparation

Gene expression of AD169-infected HEL fibroblasts was analysed at several time points after infection (0, 6, 12, 24, 48, 72 and 96 hours). Uninfected HEL cells were also included in the analysis. Total RNA was purified from all samples and the quality assessed by agarose gel electrophoresis (Figure 6.1). mRNA was purified and labelled with Cy5 using RT-PCR. Labelled cDNA was mixed with Cy3-labelled reference RNA and hybridised to the HCMV-human microarray. A common reference RNA mixture was used to permit comparison across the whole sample set (see section 4.1.2.2).

6.2.2 Analysis of gene expression

The arrays were used to monitor host gene expression in fibroblasts after infection with HCMV AD169. The data were filtered and assembled with data from Toledo-infected MRC-5 fibroblasts, to produce a set of 1477 human genes. The data were examined by cluster analysis to identify patterns in the gene expression data and identify relationships between the samples. The genes were ordered using a self-organising map algorithm, which minimises the differences between adjacent nodes. This ordering was then used to control the orientation of the nodes of the dendrograms subsequently generated by hierarchical clustering. The programme TreeView (Eisen *et al*, 1998) was used to visualise the expression of the filtered set of 1477 genes (Figure 6.1). This analysis reveals how expression of the genome varied throughout the time-course of HCMV infection and how gene expression in AD169-infected fibroblasts related to that in Toledo-infected fibroblasts. The analysis resulted in two clusters of gene expression, showing a clear distinction between the two time-courses (Figure 6.1). In turn, within each time-course, a separation between the early and late time points is evident. The data were also subjected to hierarchical clustering for both genes and samples, in order to follow the exact changes occurring throughout the time-courses of infection (Figure 6.3).

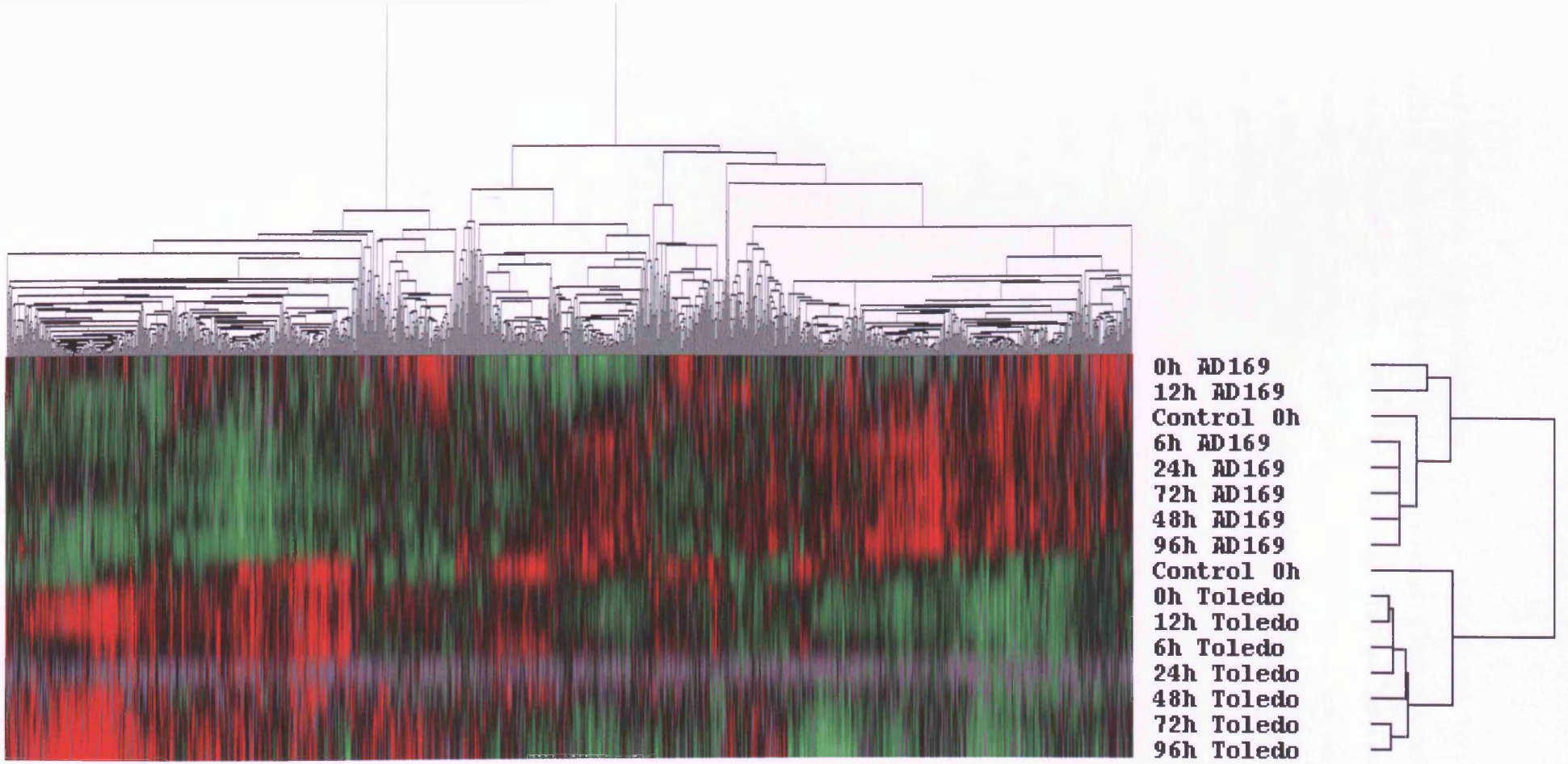

0h AD169
12h AD169
Control 0h
6h AD169
24h AD169
72h AD169
48h AD169
96h AD169
Control 0h
0h Toledo
12h Toledo
6h Toledo
24h Toledo
48h Toledo
72h Toledo
96h Toledo

Figure 6.1. Hierarchical clustering of 16 samples and a filtered set of 1477 genes. Each column represents one sample and each row one gene. The dendrogram on the left represents the relationship between genes in terms of their expression pattern. Gene expression is shown as a pseudo-coloured representation of log(2) expression ratio with red being above and green below the row/column median level of expression (set to 0) as shown by the scale.

I used microarrays to investigate the effect of AD169 and Toledo infection in fibroblasts. The analysis identified genes from a range of host cell pathways that show changes in expression levels after viral infection. Previous microarray analysis of fibroblasts infected with AD169 revealed a number of cellular genes whose expression was enhanced by the virus (Zhu *et al*, 1998, Browne *et al*, 2001). AD169 was shown to increase transcript levels of the IFN-induced 17kD protein, characteristic of an innate response to infection. The mRNA for decay accelerating factor (DAF), a complement mediator, was also up-regulated in infected cells, among others. Nevertheless, virus infection resulted in the suppression of a number of host transcripts, including ones involved in cell cycle regulation, apoptosis and cytoskeletal structure (Zhu *et al*, 1998). Our results are in agreement with these findings. Some of the genes we confirmed are shown in Figure 6.2.

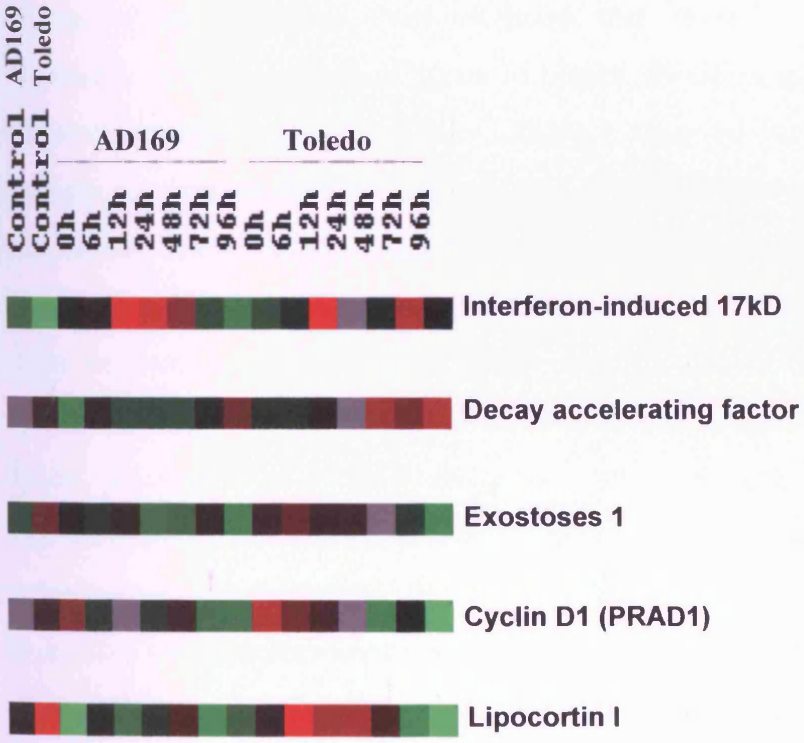


Figure 6.2. A selection of genes whose expression levels after infection of fibroblasts with HCMV confirms previous findings.

6.3 Common sets of host genes modulated by both strains

Analyses of the data suggest that the viruses induce both general and specific changes in host gene expression over a 4-day time-course (Figure 6.3). Initially, I was interested in which groups of genes possessed similar expression profiles during infection and whether sets of co-expressed genes would be functionally related. Hierarchical clustering (Eisen *et al*, 1998) of 1474 genes revealed groups of genes that were induced or suppressed in response to both virus strains at one or more points during the infection time-course. The genes were classified based on their known or putative functions and clustered according to their expression profiles. Functional classification showed that some of these genes are associated with immune responses, cell proliferation, protein transport and cytoskeletal structure.

6.3.1 Host genes induced by both strains

Microarray hybridisations have revealed that diverse RNA viruses elicit the expression of common sets of genes in plants, including genes involved in defence and stress responses (Whitham *et al*, 2003). I observed the induction of an antiviral immune response in fibroblasts, consisting of IFN-responsive genes, by both AD169 and Toledo. These include ISG20 (HEM45), tryptophanyl-tRNA synthetase (Fleckner *et al*, 1991), IFI27, HLA-C (Gobin *et al*, 1999) and IFN-gamma up-regulated, IFN-alpha induced, and IFN-induced 17kD proteins. Differential display analysis has shown that both strains strongly activate the accumulation of interferon-responsive RNAs (Browne *et al*, 2001), supporting these findings. The induction of IFN-responsive genes has also been documented after HPV-16 infection. Oligonucleotide arrays were used to investigate the effect of integration of HPV-16 on gene expression in cervical keratinocytes (Alazawi *et al*, 2002). The authors identified 85 genes that showed changes in expression levels after viral integration. Interestingly, integration was associated with up-regulation of IFN-responsive genes, in comparison with a baseline of episomally infected cells, suggesting that the physical risk of high-risk HPV may influence the response to IFN in infected keratinocytes.

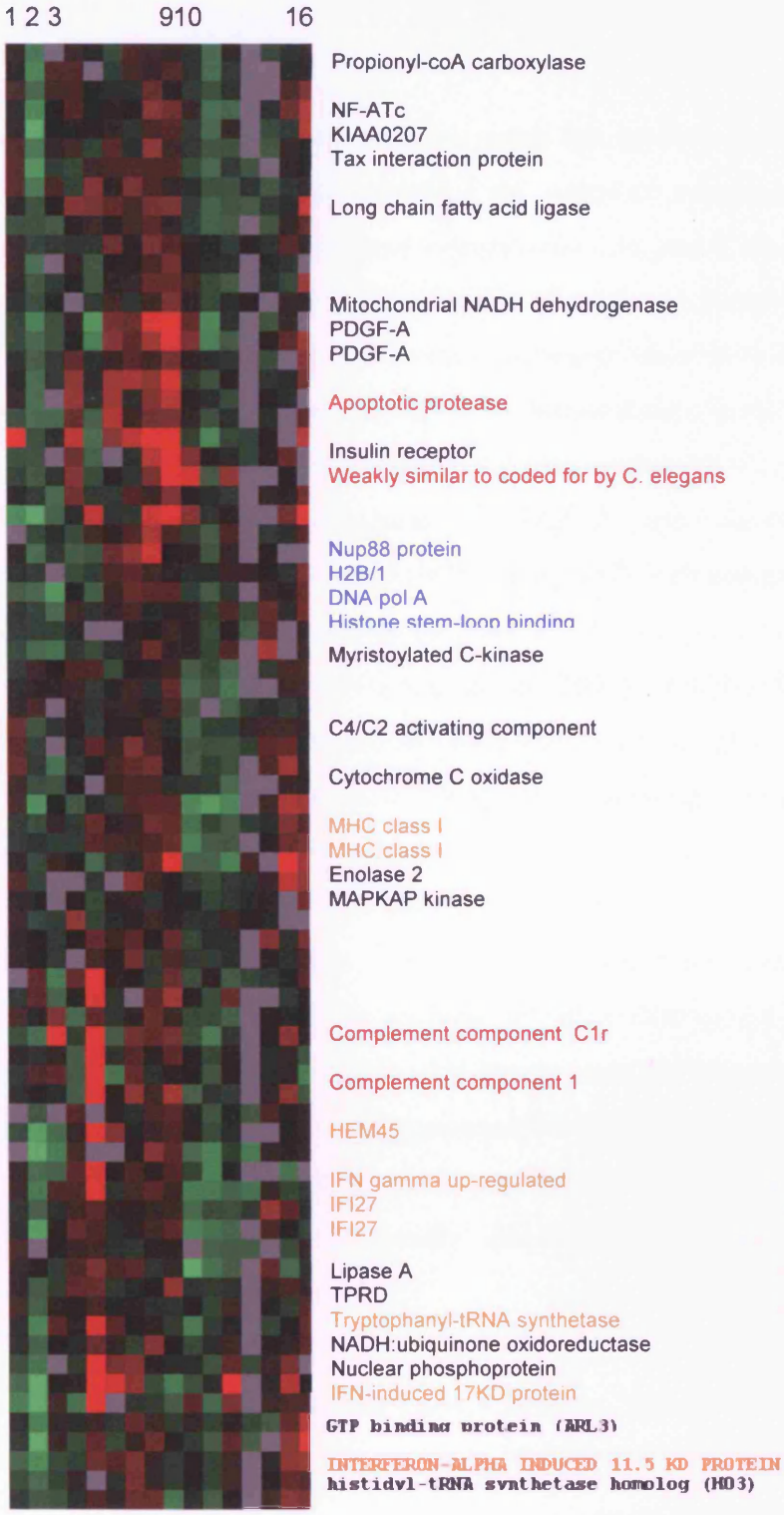


Figure 6.3. Detail showing common sets of genes induced by both AD169 and Toledo after infection of fibroblasts. This cluster is enriched for IFN-responsive genes (in orange), nuclear genes (in blue), genes involved in the complement pathway and apoptosis (in red), and ones mediating intracellular signalling (in black). Columns 1 and 2 show fibroblasts before infection. Columns 3 to 9 show fibroblasts at successive time points in hours post infection with HCMV AD169. Columns 10 to 16 show fibroblasts infected with ToledoE.

Apart from IFN-response genes, the cluster also contains genes that mediate the host antiviral response to infection. These include genes of the classical complement pathway (C4/C2 activating component, complement components C1s and C1r) and ones involved in apoptosis (apoptotic protease, KIAA0670) (Rawal and Pangburn, 2003, Schwerk and Schulze-Osthoff, 2003). Furthermore, infection of fibroblasts with the two strains led to increased levels of genes involved in intracellular signalling, such as GTP binding protein (ARL3) (Hillig *et al*, 2000) and myristoylated C-kinase, a protein kinase C (PKC) substrate. An induction of PDGF-A was observed, consistent with previous findings (Srivastava *et al*, 1999), along with an increase in GRB10 transcript levels. GRB10 has been shown to bind PDGF receptor and is essential for insulin receptor signalling (Giovannone *et al*, 2003). Additionally, increased levels of Grb10 elevate several metabolic responses such as glycogen synthesis, glucose transport, amino acid synthesis, and fatty acid processing (Deng *et al*, 2003). Interestingly, the cluster contains genes that are involved in these responses, such as TPRD, histidyl-tRNA synthetase and nuclear phosphoprotein (protein synthesis), insulin receptor and enolase (glucose uptake), along with mitochondrial genes that mediate fatty acid catabolism. The analysis revealed that genes are clustered according to their function. For example, the insulin receptor shares the same expression profile with MAPKAPK3 kinase, consistent with its role in MAPK induction. Furthermore, GRB10 clusters next to tax interaction protein 2. Both of these genes bind receptor tyrosine kinases. Additionally, this cluster contains genes that are part of closely-related cellular pathways.

Finally, the cluster contains four genes that encode nuclear proteins, such as NUP88, H2B/l, DNA polymerase alpha and histone-binding protein. NUP88 is a major protein of the nuclear pore complex (Griffis *et al*, 2003), while H2B/l functions in chromatin condensation. Gene delivery studies have suggested that exogenous DNA present in the cytoplasm is initially docked to and translocated through a nuclear pore by the nuclear import machinery. As DNA enters the nucleus, it is quickly condensed into a chromatin-like structure, which provides a mechanism for threading the remaining worm-like molecule through the pore (Zanta *et al*, 1999). Accordingly, this can be a strategy that HCMV employs in order to enter the host cell's nucleus and initiate replication. Overall, my results indicate that AD169 and Toledo both induce a number of immunoregulatory, signalling and nuclear genes. The ability of the two strains to

elicit common gene expression changes could reflect the importance of these genes to viral infection processes.

6.3.2 Host genes suppressed by both strains

Hierarchical clustering of gene expression data revealed a common set of mRNAs that were suppressed as a result of infection with either AD169 or Toledo. These consisted mainly of genes involved in cell proliferation, apoptosis and protein synthesis and transport. The expression of host genes modulated either through viral action or by host reaction involves a "struggle" between the expression of pro-apoptotic and anti-apoptotic (proliferative) molecules. There is normally a balance between apoptotic and anti-apoptotic signals, and cell death occurs in response to a persistent shift in this balance. Although the expression of various apoptotic and anti-apoptotic signalling molecules were differentially regulated in this study, their potential diverse and unknown functions make it difficult in some instances to draw strong conclusions regarding the impact of their transcriptional regulation. Of note, however, is the down-regulation of DAP-1, caspase 3, caspase 7 (Mch3) and adenylate kinase 2 after infection (Figure 6.4). The down-regulation of these molecules appears to contribute to cell survival. This may represent a host stress response aimed at cell survival or a strategy that HCMV employs in order to promote cell viability for virus replication. HCMV infection activates both proliferative and anti-proliferative signals in infected cells, depending on cell type. HCMV is known to induce non-permissive cells to enter the cell cycle, while infection of permissive cells results in cell cycle arrest. My analysis showed that transcript levels of genes that promote cell cycle progression were down-regulated, including NTAK (Nakano *et al*, 2000), cyclin I, and histone deacetylases 1 and 3 (Yoshida *et al*, 2003) among others (Figure 6.4). These results are consistent with previous findings showing a suppression of proliferative mRNAs after infection with AD169, the laboratory-adapted HCMV strain. Furthermore, they illustrate that the wild type virus may also induce these effects.

It is thought that interaction of viral proteins with the host machinery involved in the cellular transport system permits the transport of newly synthesised viral capsids in the cytoplasm (Sodeik, 2000). A recent study has identified two host proteins associated with HCMV particles, ES130 and p180 (Ogawa-Goto *et al*, 2002). ES130

is a member of the kinectin family, while p180 functions in membrane proliferation and secretion (Rezaee *et al*, 1993, Becker *et al*, 1999). The authors also reported high levels of the p180 protein in HCMV-infected cells, suggesting that the level of expression of p180 may be an essential factor for HCMV replication. The dependence of virus transport on host factors has also been demonstrated for HPV-16, where studies showed that binding to beta-actin facilitates viral transport across the cytoplasm during infection (Yang *et al*, 2003). Viruses have also been shown to interfere with the cellular transport machinery. When the effect of poliovirus infection on protein transport was studied, it was found that transport of both plasma membrane and secretory proteins was inhibited early in the infectious cycle (Doedens and Kirkegaard, 1995, Gustin and Sarnow, 2001). Hepatitis C virus was also found to inhibit ER-to-Golgi traffic (Konan *et al*, 2003). Additionally, the host cell transport machinery can be engaged differentially by different strains of a virus (Garner and LaVail, 1999).

I found several genes involved in protein synthesis and transport to be down-regulated in fibroblasts infected with either AD169 or Toledo (Figure 6.4). The majority of these genes were associated with the ER. These consist of calnexin, beta-cop, ER lumen protein retaining receptor (KDELR2), calumenin (Vorum *et al*, 1999), phosphatidylinositol-glycan and ADP-ribosylation factor 6 (ARF6). Calnexin, a gene essential for the retention of incorrectly folded proteins in the ER, clustered next to KDELR2 and beta-cop, two genes that are also involved in ER protein retention (Norkin *et al*, 2002, Lu *et al*, 2003, Oda *et al*, 2003, Yamamoto *et al*, 2003). ARF6 was suppressed in infected fibroblasts, but to a further extent in Toledo-infected cells (by a factor of 4). The potential role of ADP-ribosylation factor in vesicular trafficking was demonstrated in 1992, using an *in vitro* assay that efficiently reconstituted transport between the ER and Golgi compartment in mammalian semi-intact cells, a population of cells in which the plasma membrane is physically perforated to reveal intact ER and Golgi compartments. ARF inhibitors were shown to inhibit transport of the vesicular stomatitis virus (VSV) G protein between the ER and cis-Golgi compartment rapidly and irreversibly (Balch *et al*, 1992). These results suggested that ARF might play an important role in the cycling and fusion of transport vesicles mediating ER to Golgi trafficking.

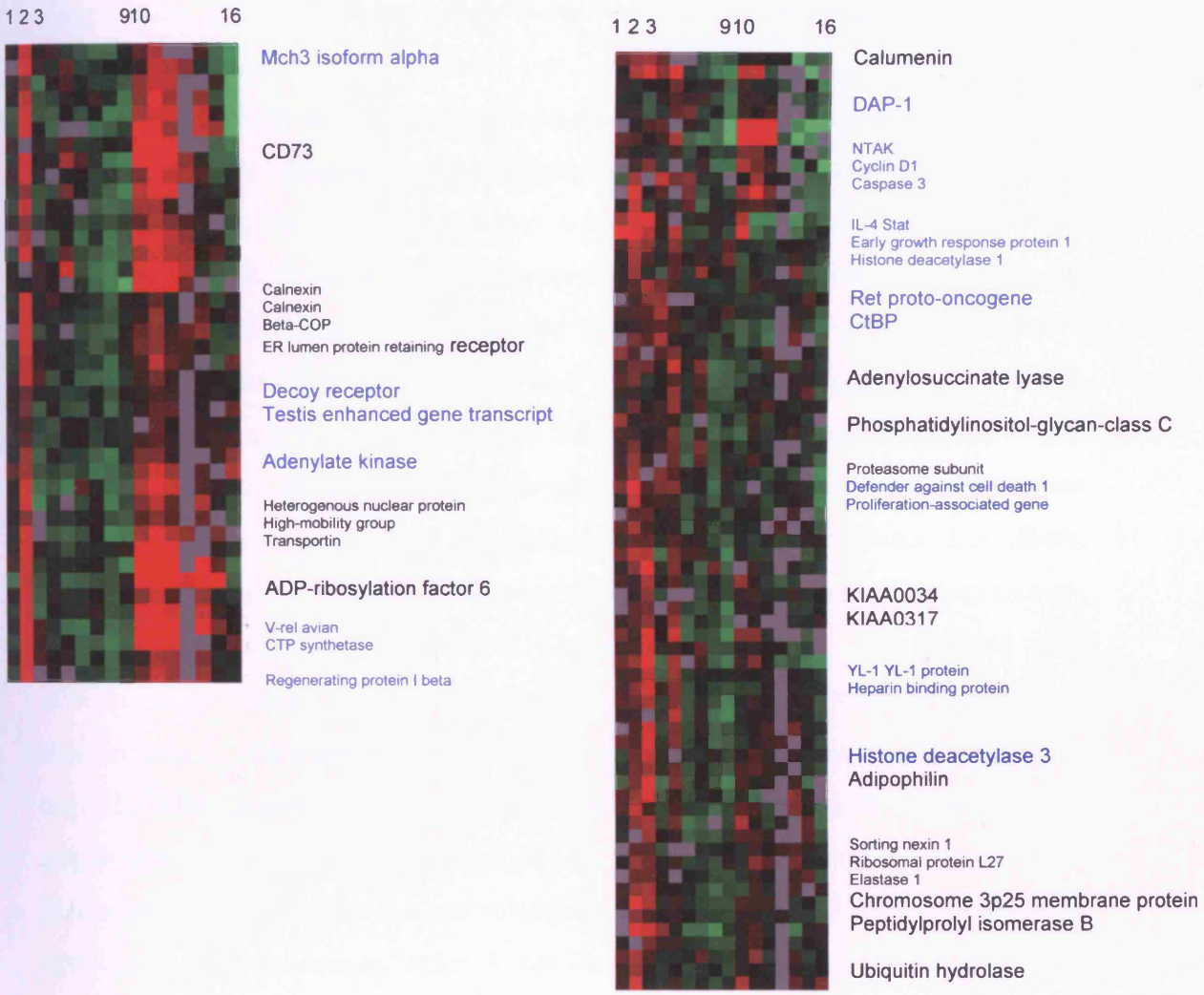


Figure 6.4. Detailed analysis, showing genes suppressed by both AD169 and Toledo after infection of fibroblasts. These clusters contain proliferative and anti-proliferative genes (in blue) and genes involved in protein synthesis and transport (in black). Columns 1 and 2 show fibroblasts before infection. Columns 3 to 9 show fibroblasts at successive time points in hours post infection with HCMV AD169. Columns 10 to 16 show fibroblasts infected with ToledoE.

6.4 Strain-specific effects on host transcription programmes

6.4.1 Host genes induced by Toledo

Many human viruses can cause chromosomal anomalies and damage to the mitotic apparatus, including herpes simplex 1 and 2, adenovirus, cytomegalovirus, Epstein-Barr virus, poliovirus and papillomavirus (Luleci *et al*, 1980, Aranda-Anzaldo, 1992, Deng *et al*, 1992, Duensing and Munger. 2002, Fortunato and Spector, 2003). Chromatid breaks, translocations and over-condensation are some of the irregularities seen. All of the above viruses cause non-specific chromosomal abnormalities, except from the oncogenic adenoviruses; infection of human cells with adenovirus type 12 has been shown to result in fragility in four common sites (Schramayr *et al*, 1990). Several reports have shown that HCMV infection causes random chromatid breaks and gaps *in vitro*. However, it was recently reported that the virus induces two specific breaks in chromosome 1, at positions 1q21 and 1q42 (Fortunato *et al*, 2000). Hierarchical clustering of the gene expression data revealed a cluster of genes that are strongly suppressed after infection of fibroblasts with AD169 but induced after infection with Toledo (Figure 6.5). Interestingly, 27 of these genes are located on chromosome 1. More specifically, 10 of the genes are located at position 1q21, the major HCMV chromosomal breakpoint (Figure 6.5A). These include: the S100 genes calgranulin A and CAAF1, involved in cell cycle regulation (Holly *et al*, 1995, Hitomi *et al*, 1998); mucin 1, a cell adhesion gene (Mitchell *et al*, 2002, Truant *et al*, 2003); adenosine deaminase, which destabilises double stranded RNA (Saunders and Barber, 2003); the mitochondrial genes HAX1 and metaxin (Bornstein *et al*, Marenholz *et al*, 2001); the guanine nucleotide regulatory factor, which is involved in the control of cell shape and size (Mayer *et al*, 2001); and the IL-6 receptor (Takashiba *et al*, 2003). The cluster also contains genes that are involved in the regulation of chromatin structure, such as KIAA0166, PHD finger 1 (Jones *et al*, 2000), CDC46 and Mi-2 protein (Ishimi *et al*, 1996) (Figure 6.5B). CHC1, a gene that regulates chromosome segregation (Nishimoto *et al*, 1994), was up-regulated by a factor of four 48 hours after Toledo infection, along with DNAS1L3, which is involved in cleavage of chromatin DNA (Yakovlev *et al*, 1999) (Figure 6.5B). These results suggest that the specific chromosomal abnormalities that are induced after HCMV infection could be the result of viral modulation of host genes.

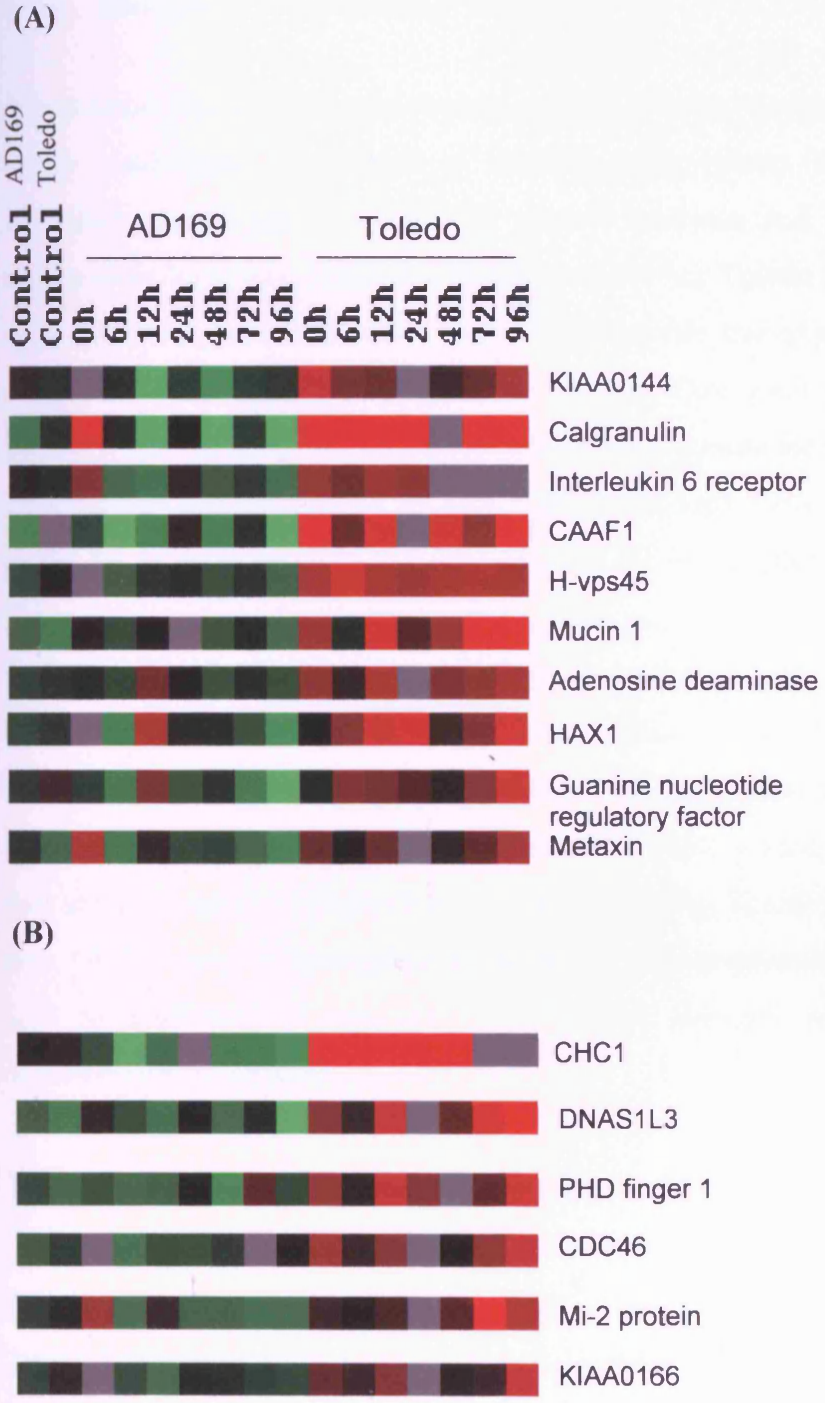


Figure 6.5. Genes that are modulated differentially after infection of fibroblasts with either AD169 or Toledo. This gene expression signature contains **(A)** genes located at chromosomal position 1q21 and **(B)** genes that are involved in the regulation of chromatin structure.

6.4.2 Host genes suppressed by Toledo

Hierarchical clustering of gene expression data revealed a cluster of mRNAs that were strongly suppressed as a result of infection with Toledo (Figure 6.6). Although I identified several genes involved in protein synthesis and transport, to be down-regulated in fibroblasts infected with either AD169 or Toledo (Figure 6.4), this cluster contains some genes involved in intracellular protein transport that are preferentially suppressed after infection with HCMV Toledo. One such gene is the ER lumen protein retaining receptor 2 (KDELR2), which is essential for the retention of luminar ER proteins and for normal vesicular traffic through Golgi (Majoul *et al*, 2001). KDELR2 is suppressed by a factor of four, 72 hours after infection with Toledo. Other genes include the clathrin coat assembly gene, involved in vesicle transport, and the ADP ribosylation factors 1 and 4, which mediate vesicle budding and uncoating within Golgi (Moss and Vaughan, 1995, Chavrier and Goud, 1999). Clathrin-associated mRNA is suppressed by a factor of three, 96 hours after Toledo infection. This gene initiates coat formation and links clathrin to receptors in coated vesicles that are involved in endocytosis and Golgi processing. These results indicate that wild type HCMV may down-regulate a number of vesicle-associated genes, which could lead to inhibition of antigen presentation at the cell surface and subsequent accumulation of intracellular virus.

The cluster also contains genes that are involved in cell adhesion and extracellular matrix composition, such as SPARC/osteonectin (Bornstein, 2002, Brekken *et al*, 2003), integrin, beta-sarcoglycan (Crosbie *et al*, 1999), and CD44 (Navaglia *et al*, 2003). Fibronectin 1 and Cyr61, two cell adhesion genes required for embryogenesis, are also suppressed after infection of fibroblasts with Toledo (O'Brien and Lau 1992, Armstrong and Armstrong, 2003). Cell adhesion is an essential process for embryogenesis. Therefore, the suppression of cell adhesion by HCMV could contribute to the development of problematic pregnancies in infected individuals.

Finally, a number of mRNAs that are highly expressed in muscle tissue are located in the same cluster (Figure 6.6). These include lysyl hydroxylase, glucan, DNAse I-like and ras-related GTP-binding proteins (Gromov *et al*, 1995, Valtavaara *et al*, 1997). Furthermore, the expression of DOC1 (putative oral tumor suppressor) and calponin

3, proteins involved in muscle contraction, is reduced by a factor of five in Toledo-infected fibrolasts (Kaneko *et al*, 2000). Overall, these results suggest that infection with wild type virus may lead to reduced contractility of the cardiac muscle, contributing to the development of cardiovascular disease in HCMV-infected immunocompromised patients.

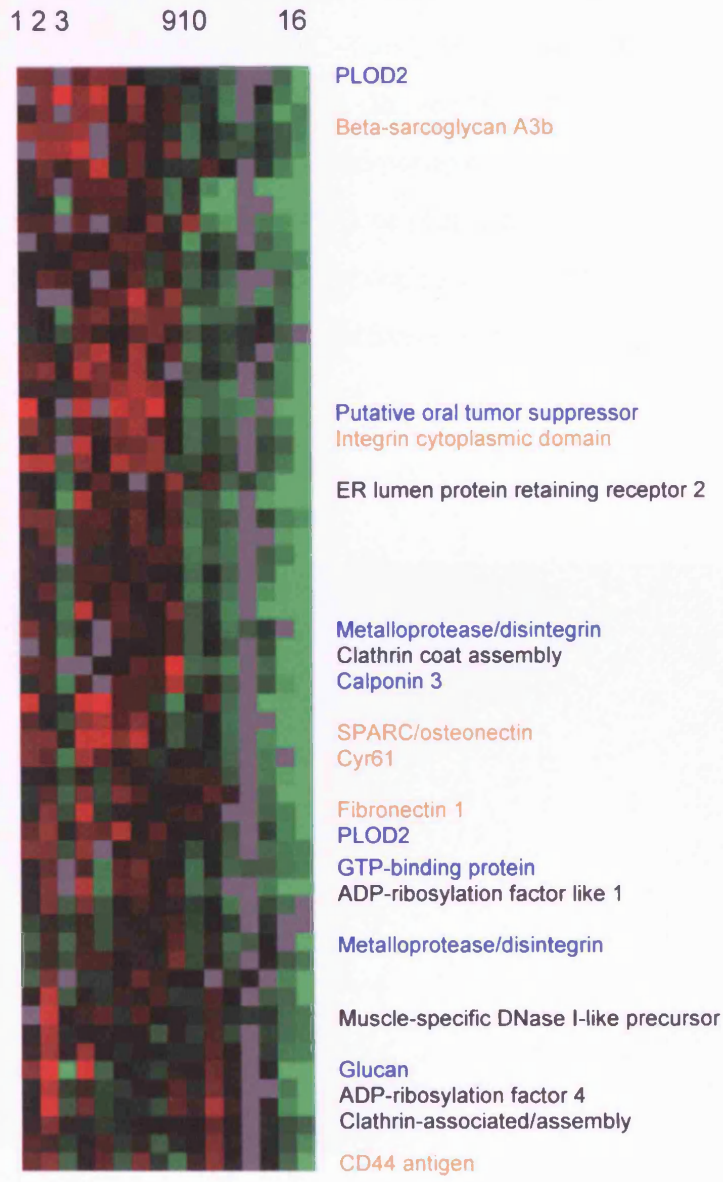


Figure 6.6. Detail showing genes that are down-regulated after infection of fibroblasts with Toledo. This cluster contains cell adhesion genes (in orange), genes involved in intracellular protein transport (in black) and ones that play a role in muscle function (in blue). Columns 1 and 2 show fibroblasts before infection. Columns 3 to 9 show fibroblasts at successive time points in hours post infection with HCMV AD169. Columns 10 to 16 show fibroblasts infected with ToledoE.

6.5 Comparison of gene expression patterns between uninfected fibroblasts

Gene expression of AD169-infected HEL fibroblasts was analysed at several time points after infection (0, 6, 12, 24, 48, 72 and 96 hours). The data were filtered and assembled with data from Toledo-infected MRC-5 fibroblasts, to produce a set of 1477 human genes. Mock-infected MRC-5 and HEL fibroblasts were also included in the study, to enable comparison with data derived from the time-course experiments. Pair-wise analysis of two normalised microarrays was performed, each hybridised with RNA derived from uninfected MRC-5 or HEL fibroblasts (Figure 6.7). Statistical analysis of the log(2) expression ratios revealed a correlation coefficient of r=0.67, indicating that fibroblasts derived from different sources have slightly different gene expression patterns.

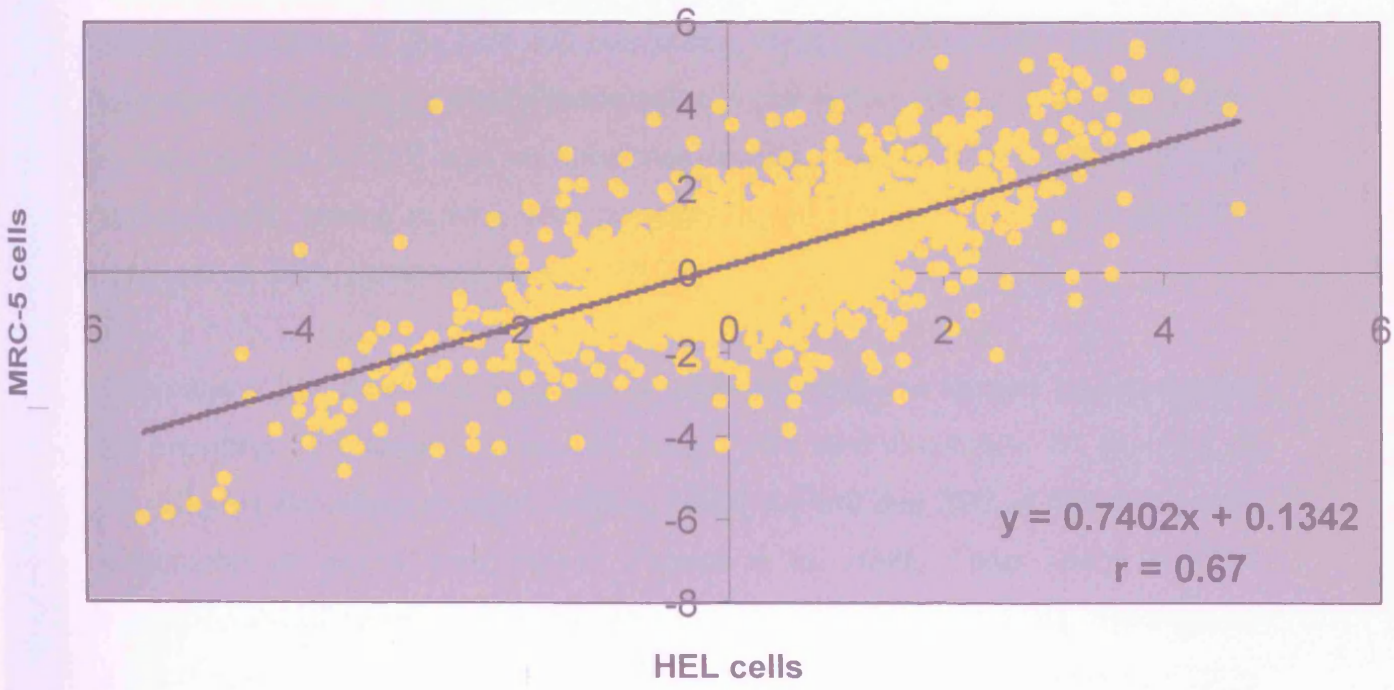


Figure 6.7. Comparison of RNA targets from MRC-5 and HEL fibroblasts (r=0.67).

6.6 Discussion

Several studies have taken place aiming to identify cellular genes that are responsible for the pathological differences observed between infections with different viruses. One such study used DNA arrays to compare the interactions between HSV-1 and HSV-2 with the cellular machinery. The results identified a group of genes that was differentially expressed during infection of cells with the two viruses, providing new information about host defence against viral infections and clues to the molecular basis for the differences between infections with HSV-1 and HSV-2 (Paludan *et al*, 2002).

Different viral strains also have different potentials to cause disease, based on the contributions of specific viral proteins to the ability of the virus to replicate and spread within the host and to escape host protective responses. Attenuation of disease potential can occur following passage of virus in cell culture in the absence of the selective pressures of the host cell machinery, tissue architecture and host immune system or by selection for rapid dissemination in cell culture (Bower *et al*, 1999). This is the case for HCMV and encephalomyocarditis virus (EMCV), which become attenuated for growth *in vivo*, after passages in cell culture (Waldman *et al*, 1991, Grefte *et al*, 1995, Denis and Koenen, 2003).

Comparison of virulent and attenuated strains of a virus can identify key genes that are important for disease pathogenesis. Arrays were used to compare the genomes of 15 different *Helicobacter pylori* isolates, which showed that 22% of the genes were dispensable in one or more strains (Salama *et al*, 2000). These genes included restriction modification genes and genes encoding cell surface proteins. Although no clinical data were available for these strains, the authors postulated that some of these genes might be associated with virulence. Spotted microarrays were also used to compare *Mycobacterium tuberculosis, Mycobacterium bovis* and different members of the family of Bacille Calmette-Guerin (BCG), the anti-tuberculosis vaccine produced by attenuating *M. bovis* (Behr *et al*, 1999). 91 genes were absent from one or more virulent strains of *M. bovis*. 38 genes were present in *M. tuberculosis* and *M. bovis* but absent in BCG strains. This suggested that the different genes missing from the BCG strains reflected a progressive adaptation of BCG to laboratory conditions

that may have impaired their ability to stimulate a durable immune response in the host. *M. tuberculosis* cDNA arrays were used to identify segments of DNA that are present in the sequenced strain but missing from clinical isolates (Kato-Maeda *et al*, 2001). Genomic deletions were detected in the clinical isolates, with varying distributions. Interestingly, strains with a large amount of deleted DNA were less likely to cause cavitary pulmonary disease than strains with fewer deletions. Since cavitary pulmonary disease is a transmissible form of tuberculosis, it was suggested that the accumulation of deletions might cause a decrease in bacterial fitness (Gillespie *et al*, 2002). Alternatively, this loss of genes may be a consequence of the adaptation to a new environment.

Reports have demonstrated that different virus strains have different effects on host expression, possibly contributing to varying disease pathogenesis. Contrary to their counterparts in HPV-16, E6 and E7 from benign HPV-6b have no effect on host gene expression (Nees *et al*, 2000). Also, *in vivo* infection by the pathogenic J3666 strain of PVM induces expression of interferon response genes, whereas the non-pathogenic strain 15 does not (Domachowske *et al*, 2002). Therefore, the host response against viral infection varies according to pathogenicity. The suppression of the interferon response by some viruses and not others may reflect differences in the replication strategy. For example, the initiation of KSHV lytic replication is accompanied by an increase in the host anti-viral response to the virus in DMVECs (Poole *et al*, 2002). Differences in host gene expression may also reflect the mechanism by which the virus counters the anti-viral response. For example, viruses can inhibit the function of interferon-response genes at the post-transcriptional level (Goodbourn *et al*, 2000).

Results to date argue that the alteration in cellular mRNA levels induced by AD169 and Toledo is identical (Zhu *et al*, 1998). However, only a limited number of genes were assayed. In this chapter we used DNA microarrays to identify global changes in host transcription induced by these two HCMV strains. The analysis identified genes from a range of host cell pathways that show changes in expression levels after viral infection, and showed that the viruses induce some common changes in host gene expression over the 4-day time-course (Figure 6.3). I observed the induction of an antiviral immune response in fibroblasts, consisting of IFN-responsive genes, by both AD169 and Toledo. Additionally, the analysis revealed a common set of mRNAs that

were suppressed as a result of infection with either AD169 or Toledo, consisting mainly of genes involved in cell proliferation, apoptosis and protein synthesis and transport.

Interestingly, the analysis revealed that the laboratory and clinical isolates of HCMV alter cellular gene expression differentially. Hierarchical clustering of gene expression data revealed a cluster of genes that are strongly suppressed after infection of fibroblasts with AD169 but induced after infection with Toledo (Figure 6.5A). Interestingly, 27 of these genes are located on chromosome 1. More specifically, 10 of the genes are located at position 1q21, which was shown to be the major HCMV chromosomal breakpoint, along with 1q42 (Fortunato *et al*, 2000). Fortunato *et al* identified a number of genes that are located in these breakpoints. Overall, my findings suggest that the specific chromosomal abnormalities induced after HCMV infection could be the result of viral modulation of host genes. Furthermore, the cluster also contained GNPAT (cDNA for dihydroxyacetone), a gene located at 1q42. It has been shown that defects in this gene can result in cataract and mental retardation (Burdette *et al*, 1996). Therefore, the deletion of this gene because of HCMV-induced breakage could potentially be linked to the development of the mental retardation observed in infants congenitally infected with the virus.

After the initiation of viral genomic replication in the cell nucleus, progeny capsids are assembled and bud into the perinuclear space after acquiring a primary envelope. The subsequent maturation is a poorly understood process. Reports have shown that nucleocapsids are released into the cytoplasm and then acquire final envelopment by budding into a cytoplasmic compartment (Severi *et al*, 1988, Radsak *et al*, 1996, Sanchez *et al*, 2000). The mechanism of capsid transport in the cytoplasm, however, is entirely unknown. Since movement of viral particles through the cytosol is not likely to occur by free diffusion but rather most likely by the cellular transport system, it is most probable that interaction of viral proteins with the host machinery involved in the cellular transport system permits an active process (Sodeik *et al*, 2000). Interestingly, the array analysis revealed a number of mRNAs that were preferentially down-regulated by HCMV Toledo (Figure 6.6), with some of them required for intracellular protein transport. Inhibition of the protein transport machinery by wild

type HCMV could be a strategy that the virus employs to prevent the onset of antiviral responses.

It is thought that mutations in cellular sarcomeric proteins can cause hypertrophic cardiomyopathy (HCM), a disease characterised by impaired cardiac myocyte contractility (Marian *et al*, 1997). Additionally, tissue culture experiments have shown that coxsackie virus replication in the myocardium can impair cardiac contractile function and lead to dilated cardiomyopathy (Morita *et al*, 1984, Fujioka and Kitaura, 2001). HCMV infection in the setting of heart transplant and HIV patients is associated with the development of cardiovascular disease (Li *et al*, 1996). The analysis of gene expression data from HCMV-infected fibroblasts indicated that infection with Toledo led to a reduction in the expression of mRNAs that are highly expressed in muscle and are involved in muscle contractility. Infection with AD169 did not have the same outcome. My findings are in agreement with published data from Filippov *et al* (Filippov *et al*, 2001). The authors of the study assessed the condition of the cardiovascular system in HIV-infected patients with HCMV infection, showing that HCMV affected the morphologic and functional parameters of the cardiac muscle, with one evident symptom being low myocardial contractility. I suggest that one or more of the UL/b' genes may be involved in the modulation of host genes involved in muscle function, leading to reduced contractility of the cardiac muscle, potentially contributing to the development of cardiovascular disease.

It has been reported that atherosclerotic lesions contain genomic material belonging to HCMV, suggesting a viral etiology for the development of atherosclerosis. Additionally, infection of endothelium with HCMV induces increased expression of adhesion molecules, resulting in an increased adherence of leukocytes, which is suggested to be one of the earliest events associated with endothelium cell damage (Span *et al*, 1991, Shahgasempour *et al*, 1997, Knight *et al*, 1999). We found a differential modulation of cell adhesion molecules in fibroblasts. Analysis of gene expression data revealed a suppression of these molecules after infection with Toledo. This modulation can have profound effects in the process of embryogenesis. During placental development, specialised cells called cytotrophoblasts, differentiate into the trophoblast population of floating villi and anchoring chorionic villi (Damsky and Fisher, 1998). The cytotrophoblasts of the anchoring villi then aggregate into columns

and invade the endometrium and the first third of the myometrium. By midgestation, invasive cytotrophoblasts have completely replaced the endothelial lining and much of the smooth muscle wall of these arteries, forming a hybrid vasculature, composed of fetal and maternal cells. During placentation, a molecular differentiation program is initiated, that is required for normal pregnancy (Fisher *et al*, 1993, Cross *et al*, 1994, Norwitz *et al*, 2001). For example, invading cytotrophoblasts in the anchoring villi begin to express adhesion molecules and proteinases required for invasion, such as integrin and matrix metalloproteinase 9 (Maidji *et al*, 2002). My results suggest that low-passage but not laboratory-adapted virus, induces a shut down in the transcription of such molecules, which could be mediated by the UL/b' region genes. For example, the RNA expression of metalloprotease was decreased by 12 hours after infection. This down-regulation could lead to decreased invasiveness of cells during placentation, leading to the development of pregnancy abnormalities that manifest in HCMV-infected individuals. Indeed, in support of my data, previous findings have demonstrated that HCMV infection of purified cytotrophoblasts from first-trimester placentas led to a down-regulation of integrin, and impaired ability of these cells to invade Matrigel in an *in vitro* invasion assay (Fisher *et al*, 2000).

The *in vitro* plan outlined in this chapter, has enabled the charting of prominent patterns of gene expression in HCMV-infected fibroblasts and the separation of varied and potentially significant gene expression events. Interestingly, analysis of the gene expression data revealed a Toledo-specific down-regulation of host genes, due to unique genes in the UL/b' region, which could explain Toledo's increased pathogenicity *in vivo*.

Chapter 7
General discussion and directions for future research

The interactions between viruses and the cells they infect are complex and multifaceted. While viruses strive to take over cellular functions to their advantage, the cell strives to impede these efforts by mounting a variety of defensive responses. These responses may include the induction of interferon, stress responses, or apoptotic pathways, all of which are accompanied by changes in gene expression. Some viruses consistently win this war while others succumb to cellular defence mechanisms. The viral and cellular factors that determine the outcome are mostly still unknown. With the advent of functional genomics, potent new technologies are now available to analyse the complexities of virus-host interactions in ever increasing depth and detail. I illustrate here efforts to use microarrays to focus on the changes in gene expression that occur in HCMV-infected cells and to use these technologies to unlock some of the mysteries of virus-host interactions.

In this thesis I have described the creation of DNA arrays and their use in the analysis of HCMV and host gene expression in fibroblasts and endothelial cells. The HCMV-human array shows reproducible measurements of expression both within arrays and between arrays. The arrays are specific and provide reproducible measurements of expression. I have demonstrated that array analysis reveals the transcriptional changes that control HCMV replication and the relationship between HCMV and the transcriptional machinery of the host. I have created a DNA array to identify the transcription profile of a region of the HCMV genome thought to be crucial for disease pathogenesis *in vivo*, which will enhance our understanding of HCMV behaviour. The array also contains probes for over 5,000 human genes, allowing concomitant measurements of gene expression for both host and virus. The results from this type of study can unravel interactions between the virus and host and give an insight into disease pathogenesis.

One of the factors required for successful viral replication is the ability to gain access into a susceptible cell. HCMV infection begins by the low affinity attachment of the virus on the cell, followed by high affinity attachment, fusion of the viral envelope with the cell membrane, uncoating of the nucleic acid and transport of the viral DNA to the cell nucleus. Findings suggest that annexin II, a 30kD member of the lipocortin family, is the principal HCMV receptor involved in virus binding to cells and cell to cell spread (Wright *et al*, 1994, Bold *et al*, 1996). However, another group using a different cell type, demonstrated that neutralisation of cell surface annexin II was inconsequential for direct cell to cell spread of the virus (Pietropaolo and Compton, 1999). It is therefore possible that the role of annexin II in HCMV entry, as well as the entry mechanism itself, differs from cell type to cell type. Therefore, the extent of

genome expression in any given cell type shapes the pattern of the virus-host interaction. It was illustrated recently, that EGFR is a necessary receptor for HCMV (Wang *et al*, 2003). Wang *et al* showed that HCMV infects cells through interaction of viral gB with EGFR, triggering an intracellular signalling cascade. During HCMV lytic replication viral genes are expressed sequentially in three phases, termed immediate early (IE or α), early (E or β) and late (L or γ), according to the timing of transcription (Table 1.2). HCMV replication in a particular cell type is dependent on the virus strain. *In vitro* studies have shown that endothelial cells are not generally permissive for infection with high-passage strains of HCMV (Friedman *et al*, 1981). However, these cells are permissive for infection with some low passage HCMV isolates (Ho *et al*, 1984, Waldman *et al*, 1989). Similarly, monocyte-derived immature dendritic cells were shown to support the full replication cycle of endothelial cell tropic strains of HCMV *in vitro*, resulting in the expression of IE, E and L genes and production of infectious virus (Riegler *et al*, 2000).

Different HCMV strains also display biological differences, the genetic basis for which is still being investigated. AD169, Towne and Toledo are HCMV strains commonly used in the laboratory environment. Clinical data have demonstrated that HCMV strains exhibit different levels of virulence depending on their passage history in cell culture. Extensive comparisons of the restriction enzyme profiles of Toledo and a highly passaged variant of Towne revealed an additional region of sequence at the right edge of the Toledo UL component (Cha *et al*, 1996), termed UL/b' region and encoding 19 ORFs. Although these genes are not essential for replication *in vitro*, their maintenance in clinical isolates suggests that the encoded proteins are important for HCMV pathogenesis *in vivo*. The importance of fitness determinations became evident in 1976, when it was proposed that the infecting strain of HCMV is important for clinical outcome, along with the intensity and duration of viral replication. High and low passage HCMV strains exhibit tropism differences *in vitro*, suggesting that different tissue tropism may occur *in vivo*. Indeed, differences in the distribution of HCMV infection in leukocytes from tissues of patients have been demonstrated (Sinzger and Jahn, 1996). Furthermore, reports have shown that different clinical isolates obtained from transplant patients had specific tropisms for different cells (Torok-Storb *et al*, 1993), suggesting that these different tropisms might account for differences in pathogenicity.

One of the aims of this thesis was to determine the replication dynamics of HCMV strains AD169 and Toledo *in vitro* as well as compare the ability of AD169 and Towne to bind to cells and mediate cell-to-cell spread of infection using pair-wise competition experiments in cell

culture (Chapter 3). Assessment of viral replication using quantitative PCR measure of virus over time for each of these strains, showed that AD169 replicated better than ToledoE in fibroblasts, reaching $1.4x10^9$ gB copies/μg cellular DNA, compared to $8x10^7$ gB copies/μg cellular DNA of ToledoE. Furthermore, assessment of the replication of ToledoE in a different cell type, HUVEC, indicated that the virus replicated to higher levels in fibroblasts. However, the doubling times of ToledoE in fibroblasts and HUVEC were 6.9 and 7.3 hours, respectively, suggesting that infected fibroblasts and endothelial cells can replicate their DNA at comparable rates but that the amount of virus ultimately produced is different. Towne was found to bind to HEL cells with higher affinity compared to AD169. Overall, my results indicate that fitness between AD169 and Towne is variable and dependent upon the status of the virus inoculum. When using a cell-free virus inoculum, AD169 outgrew Towne within 17 days of culture, with a fitness gain of 24%. When cell-associated virus was used as the inoculum, Towne predominated in both cells and supernatants, indicating that it is more efficient in mediating cell-to-cell spread of infection and releasing virus into the medium. Cell-to-cell spread may be important *in vivo*, and under such conditions Towne appears to be fitter. Since these experiments are difficult to perform and the results likely to reflect differences in viral and host cell parameters, I decided to investigate the use of microarrays to assess viral and cellular transcription patterns using distict HCMV strains and distinct cell types.

The experiments discussed in the first part of chapter 4 provide important controls for the biological interpretation of the array-based expression measurements that take place in the second part of chapter 4 and in chapters 5 and 6. The HCMV-human array was used to examine (i) HCMV gene expression during active replication of HCMV in fibroblasts and endothelial cells, (ii) host-specific responses to HCMV Toledo and (iii) host responses to different strains of the virus. The expression of the majority of the genes within the HCMV UL/b' genes has not been previously investigated. The HCMV-human array and RT-PCR were used to analyse expression of these genes during active replication in fibroblasts and endothelial cells. My analysis shows that genes belonging to the same functional group have similar expression profiles and has helped to assign functions to genes for which there is no available information. The classification of the three basic groups of transcripts is generally based however on measurement of transcript abundance in infections where virus-induced DNA and protein synthesis are blocked with metabolic inhibitors. Although it would be useful to monitor expression with inhibitors of translation and herpesvirus DNA polymerase, these disturb the system and, in the case of the inhibition of translation, have wide-ranging effects on the host cell. My results show that the HCMV transcriptional profile changes as the virus progresses

through the life cycle, with individual genes having distinct expression kinetics. The majority of the UL/b'genes (UL137, UL138, UL141, UL144, UL145, and UL144-UL150) exhibited the same transcription patterns in both cell types. There was no detectable expression of UL135 and UL143 during infection, consistent with the most recent predictions of Davison *et al* based on comparisons between the gene coding content of human and chimpanzee cytomegalovirus, while UL142 was expressed only in HUVEC. The remaining genes (UL133, UL134, UL136, UL139, UL140, UL151) were transcribed with delayed kinetics in fibroblasts compared to HUVEC (Mann Whitney, $p<0.05$). This data suggest that these viral genes are differentially regulated at the level of transcription and may have distinct functions in the virus life cycle.

The HCMV array could be used to screen for expression of viral and host genes in other types of endothelial cells infected *in vitro*. Additionally, the identification of groups of co-regulated genes by cluster analysis could reveal the genes that are transcribed in monocytes during latency. Once the arrays have identified such genes, other techniques could then be used to verify expression of these genes. Furthermore, the analysis of the promoter sequences of each group of genes could provide information on the regulation of the HCMV genome.

To understand how HCMV interacts with the host cell, it will be important to find which HCMV genes are expressed *in vivo*. Expressed genes could be identified by RT-PCR or DNA array analysis of amplified RNA. In addition, the synchronized analysis of host gene expression during HCMV infection can provide useful knowledge on how the viral and host genomes interact to direct the fate of the host cell.

Infection with HCMV results in complex interactions between viral and cellular factors, which perturb many cellular functions. HCMV is known to target the cell cycle, cellular transcription and immunoregulation and it is believed that this optimises the cellular environment for viral DNA replication during productive infection or during carriage in the latently infected host. Detection of the cellular transcription pathways that are activated or repressed during viral infection can lead to a better understanding of the virus-host interaction and to the discovery of new targets for therapy. The aim of chapter 5 was to use the HCMV-human microarray to identify fibroblast and endothelial cell responses to HCMV infection. To date, there have been no reports comparing the effects of HCMV in fibroblasts and endothelial cells, two cell types that clearly play a pivotal role *in vivo* during HCMV infection. In addition, most studies have used the laboratory strains of HCMV, which may not be fully representative of the wild type virus. In this study I have identified similarities and differences in viral and host transcriptional

patterns after infection of fibroblasts and endothelial cells with a low passage strain of HCMV, using DNA microarrays.

Gene expression patterns of HCMV-infected cells were analysed at several time points after infection (0, 6, 12, 24, 48, 72 and 96 hours). Two types of cells were used, MRC-5 fibroblasts and human umbilical vein endothelial cells (HUVEC). The programme TreeView (Eisen *et al*, 1998) was used to visualise the expression of the filtered set of 1746 genes (Figure 5.3). This reveals how expression of the genome varies throughout the time-course of HCMV infection and how gene expression in infected fibroblasts relates to that in infected endothelial cells. The analysis resulted in two clusters of gene expression, showing a clear distinction between the two time-courses (Figure 5.3). In turn, within each time-course, a separation between the early and late time points is evident. A number of gene expression signatures can be identified in the data. Clustering of data from the time-courses in fibroblast and endothelial cells indicated that the levels of certain classes of host genes were notably affected in response to HCMV infection. mRNAs modulated by the virus include ones involved in the complement cascade, coagulation, inflammation, apoptosis, cell cycle, interferon response, transcription, cytoskeleton, cell adhesion, tumorigenesis and mitochondrial function. I identified common pathways of host gene expression in fibroblasts and endothelial cells. These included a decrease in transcription and signal transduction, an inhibition of apoptosis and cell cycle progression, an increase in overall MHC-I transcription and the induction of an interferon-based antiviral response. Interestingly, differences in gene expression between the two cell types occurred even before HCMV infection. Increased coagulation, pro-thrombotic activity, prostaglandin synthesis and induction of mitochondrial-associated genes were only identified in endothelial cells. Other endothelial-specific responses included strong down-regulation of MHC-II, NF-kB and complement pathways. Previous studies have shown that infection of fibroblasts with AD169 suppresses caspases 1, 2 and 8 (Browne *et al*, 2001, Skaletskaya *et al*, 2001). I found caspases 4 and 7 to be significantly down regulated by Toledo in both fibroblasts and endothelial cells (at 72 and 48h pi respectively), indicating an interference of the virus in the caspase cascade at two additional positions. This manipulation of various cellular genes by HCMV in order to prevent apoptosis, could be a way by which the virus persists in the infected host. HCMV can cause cell cycle arrest, depending on the state of the cell. Quiescent fibroblasts infected *in vitro* with AD169 start synthesising several enzymes required for DNA replication. This activation however does not seem to lead to cell division, but in G1/S arrest. This virally-induced block can take place at multiple points in the cell cycle, depending on the phase of the cell cycle at which infection (Jault *et al*, 1995, Biswas *et al*, 2003). It seems that the virus creates a

favourable environment for its own replication, at the host's expense. A number of cyclins are regulated by HCMV, including cyclin B, D3, E2 and G1 (Salvant *et al*, 1998, Browne *et al*, 2001). I identified a number of genes required for cell cycle progression, that were suppressed in fibroblasts and endothelial cells at late time points after infection. A number of genes associated with host trascription, translation and signal transduction showed a decrease in their expression after infection of fibroblasts and endothelial cells with HCMV. These cluster together with genes required for maintenance of cytoskeletal structure and cell cycle progression.

A link between HCMV infection and atherosclerosis has been suggested by experimental, clinical and epidemiological studies (see section 1.9.5). I observed a reduction of anti-coagulants transciption in endothelial cells (Figure 5.7), including members of the plasminogen subfamily. The virus was also shown to up-regulate the expression of two genes that contribute to the acceleration of coagulation; phospholipid scramblase, which plays a central role in the initiation of fibrin clot formation (Basse *et al*, 1996) and placental bikunin, an inhibitor of plasminogen and hepatocyte growth factor proteins (Hamasuna *et al*, 2001). This combination of events in HCMV-infected endothelial cells may contribute to the increased coagulation observed in infected individuals and promote the development of atherosclerosis. The biological consequences of these findings at a transcriptional level require investigation through *in vitro* and *in vivo* studies.

In the setting of immunosuppression, cytomegalovirus can affect both the central and peripheral nervous systems. Neurological manifestations of HCMV infection include encephalitis, hearing loss, mental retardation, ventriculitis, myelitis, retinitis and peripheral neuropathies (Maschke *et al*, 2002). Defects in the pro-a2 chain of collagen type XI (col11a2) are the cause of a skeletal dysplasia, which is accompanied by severe hearing loss (Vikkula *et al*, 1995, McGuirt *et al*, 1999, De Leenheer *et al*, 2001). Furthermore, defects in the cDNA for dihydroxyacetone (GNPAT) cause severe growth and mental retardation, retinal pigmentary degeneration, sensorineural deafness (Burdette *et al*, 1996). The levels of these RNAs were reduced considerably after infection of endothelial cells with Toledo, which could contribute to the development of HCMV-associated diseases (Figure 5.8).

TLR2 recognizes HCMV virions and triggers inflammatory cytokine production, mediated via TLR2-dependent activation of NF-kB (Compton *et al*, 2003). Interestingly, I observed a suppression of TLR2 transcription after infection of endothelial cells but not in fibroblasts. A decrease in TLR2 levels in response to HCMV infection could lead to viral persistence in endothelial cells, by inhibiting the NF-kB mediated inflammatory response. Consistent with a suppression of the activation of the NF-kB pathway in endothelial cells, a down-regulation of several other molecules involved in the activation of this pathway was observed. These findings suggest that HCMV infection prevents external signalling to the cell in order to optimise the cellular environment for virus replication.

I found that the low passage strain of HCMV, Toledo, strongly induced the accumulation of IFN-responsive RNAs very early during its replication cycle in endothelial cells (6-12h pi) (Table 5.1). Interestingly, the induction was weak and delayed in fibroblasts. A startling feature of HCMV infection is how the host can be tricked into committing its immune response to aiding virus dissemination. For example, my results indicate that the virus affects IFN signalling differentially in fibroblasts and endothelial cells, mounting a vigorous response in the latter cell type. One cluster of genes that are strongly induced in endothelial cells after HCMV infection is enriched for those with known anti-viral properties or that are induced by interferon (Figure 5.10A). The anti-viral cluster contains genes that act within cells to inhibit virus replication, such as IRF-1, IFI27 and STAT1 (Samuel, 2001). Overall, these results indicate that the virus affects IFN signalling differentially in fibroblasts and endothelial cells, mounting a vigorous response in the latter cell type. This could be a tactic that HCMV employs in order to reduce its replication levels, facilitating its long-term association with the host.

This anti-viral cluster also contains four inflammatory genes, two of which mediate prostaglandin synthesis (Broberg *et al*, 2002, Haeggstrom *et al*, 2002, Sherratt *et al*, 2003, Zaitsu *et al*, 2003) (Figure 5.10C). It has been shown that the synthesis of prostaglandin E2 is activated by the induction of cox-2 in HCMV-infected fibroblasts and that prostaglandin inhibitors can reduce HCMV replication levels (Zhu *et al*, 1998). I observed increased accumulation in prostaglandin synthesis in HUVECs compared to fibroblasts, occurring at just 1 hour after infection (leukotriene A4 hydrolase, microsomal glutathione S-transferase) (Figure 5.8C). This could have significant clinical applications, since it suggests that inhibitors may have differential effects in different cells in the body. Future experiments could comprise infectivity assays (plaque assay, IFA, PCR etc), in which fibroblasts and HUVEC would be

infected with Toledo and subsequently a prostaglandin inhibitor (ie aspirin) would be added at increasing concentrations. In this case, the IC_{50} should be lower in HUVEC.

The analysis also shows that HCMV Toledo induces up-regulation of CD46 and CD55 in both fibroblasts and HUVEC, but to higher levels in the latter cell type. Additionally, the complement components 1, C2 and 8 were suppressed at 6h after infection (Figure 5.11B) only in HUVEC. Overall, the inhibition of the complement cascade was more prominent in endothelial cells than in fibroblasts. The down-regulation of complement proteins in endothelial cells has not been shown previously and represents a unique strategy of HCMV for modulation of the complement cascade. The circumvention of complement attack can lead to persistence of HCMV-infected endothelial cells in the circulation and subsequent spread of the virus to different sites in the body.

There is substantial genetic heterogeneity not only among different laboratory strains, but also between laboratory strains and clinical isolates, and genetic differences between human and animal strains are profound. Given these genetic differences, one would anticipate differences in biological activity between strains. Comparison of virulent and attenuated strains of a virus can identify important genes that are important for disease pathogenesis. Results to date argue that the alteration in cellular mRNA levels induced by AD169 and Toledo is identical (Zhu *et al*, 1998). However, only a limited number of genes were assayed in these studies. In chapter 6, DNA microarrays were used to identify global changes in host transcription induced by these two HCMV strains. The analysis identified genes from a range of host cell pathways that show changes in expression levels after viral infection, and showed that the viruses induce some common changes in host gene expression over the 4-day time-course (Figure 6.3). The induction of an antiviral immune response was observed in fibroblasts, consisting of IFN-responsive genes, by both AD169 and Toledo. Additionally, the analysis revealed a common set of mRNAs that were suppressed as a result of infection with either AD169 or Toledo. These consisted mainly of genes involved in cell proliferation, apoptosis and protein synthesis and transport.

Interestingly, my analysis revealed that the laboratory and clinical isolates of HCMV alter cellular gene expression differentially. Since the genetic differences between these isolates are essentially the presence of the UL/b' region, these differential effects may give clues to the function of these gene products. Hierarchical clustering of gene expression data revealed a cluster of genes that are strongly suppressed after infection of fibroblasts with AD169 but

induced after infection with Toledo (Figure 6.5A). Interestingly, 27 of these genes are located on chromosome 1. Overall, my findings suggest that the specific chromosomal abnormalities induced after HCMV infection could be the result of viral modulation of host genes. Interaction of viral proteins with the host machinery involved in the cellular transport system permits an active process (Sodeik, 2000). The array analysis also revealed a number of mRNAs that were preferentially down-regulated by HCMV Toledo (Figure 6.6), with some of them required for intracellular protein transport (KDELR2, clathrin coat assembly gene and ADP ribosylation factors 1 and 4). Inhibition of the protein transport machinery by wild type HCMV could be a strategy that the virus employs to prevent the onset of antiviral responses. HCMV infection in the setting of HIV patients is associated with the development of cardiovascular disease (Li *et al*, 1996). The analysis of gene expression data from HCMV-infected fibroblasts indicated that infection with Toledo led to a reduction in the expression of mRNAs that are highly expressed in muscle and are involved in muscle contractility. Infection with AD169 did not have the same outcome, suggesting that the UL/b' genes could be responsible for this outcome. I propose that modulation of host genes involved in muscle function by HCMV could lead to the reduced contractility of cardiac muscle, potentially contributing to the development of cardiovascular disease.

Differential modulation of cell adhesion molecules in fibroblasts was also observed. Analysis of gene expression data revealed a suppression of these molecules after infection with Toledo. This modulation can have profound effects in the process of embryogenesis. The results suggest that low passage but not laboratory-adapted virus, induces a shut down in the transcription of such molecules. For example, the expression of metalloprotease was decreased by 12 hours after infection. This down-regulation could lead to decreased invasiveness of cells during placentation, leading to the development of pregnancy abnormalities that manifest in HCMV-infected individuals. Indeed, in support of this data, previous findings have demonstrated that HCMV infection of purified cytotrophoblasts from first-trimester placentas led to a down-regulation of integrin, and impaired ability of these cells to invade Matrigel in an *in vitro* invasion assay (Fisher *et al*, 2000). However, on the other hand, this down-regulation could be a way of allowing infected cells to stop adherence and therefore traffic around the body to initiate new infections.

Since the first isolation of HCMV in 1956, this virus has been routinely passaged in fibroblast cell cultures. However, several pieces of evidence suggest that the resulting combinations of viral strains/cell lines are not faithful paradigms of disease pathogenesis in humans. First,

HCMV is tropic for endothelial cells, monocytes and multiple cells of epithelial cells in origin *in vivo* leading to hepatitis, retinitis, pneumonitis, enteritis and a possible contribution to atherosclerosis. Second, these diseases occur with different frequencies in distinct groups of infected patients, implying a host contribution to viral pathogenesis. Third, HCMV strains passaged in fibroblasts *in vitro* lack approximately 22 open reading frames, which encode putative pathogenicity genes. In this thesis I have explored the use of gene arrays to examine expression of these novel viral genes in endothelial cells in parallel with expression in fibroblasts by means of amplification of viral DNA to overcome the inherent low multiplicity of infection of HCMV *in vitro*. The results also demonstrate modulation of distinct host genes in these two cell types and so identify candidate genes of viral and cellular origin, which may interact during natural infection to cause disease in distinct patient populations. Furthermore, they illustrate how gene array technology may be applied to bypass cellular restrictions imposed by the current inability to propagate authentic target cells *in vitro*, and so may be relevant for studies of other human viral pathogens. Unravelling the complex interplay between HCMV, the host cell and the whole organism will ultimately lead to improved understanding of viral pathogenesis and open up new avenues for therapeutic control.

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HCMV clone sequences

The identity of each cloned array probe spotted on the HCMV array was confirmed by PCR with probe-specific primers (Figure 4.1) and DNA sequencing (see section 2.3.6). Cloned array probes were also checked for the possibility of cross-hybridisation, both against each other and against host genes, by searching GenBank using pair-wise sequence analysis (www.ncbi.nlm.nih.gov/BLAST).

>clone UL132

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CCCTTATCAGCGGTTGGAAACTCGGGACTGGGACGAGGAGGAGGAGGCGTCCGCGGCCCGCG
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>cloneUL136

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>cloneUL133

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>cloneUL134

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>cloneUL135

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>clonegB

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